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The Biological Bulletin accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). Very short papers (less than 10 manuscript pages including tables, figures, and bibliography) will be published in a separate section entitled “Short Reports.” A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

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 HUETTNER, DR. AND MRS. ROBERT J.
 HUTCHISON, MR. ALAN D.
 HYNES, MR. AND MRS. THOMAS J., JR.
 INOUÉ, DR. AND MRS. SHINYA
 ISSOKSON, MR. AND MRS. ISRAEL
 JACKSON, MISS ELIZABETH B.
 JAFFE, DR. AND MRS. ERNST R.
 JANNEY, MRS. F. WISTAR
 JEWETT, G. F., FOUNDATION
 JEWETT, MR. AND MRS. G. F., JR.
 JONES, MR. AND MRS. DEWITT C., III
 JONES, MR. AND MRS. FREDERICK, II
 JONES, MR. FREDERICK S., III
 JORDAN, DR. AND MRS. EDWIN P.
 KAAH, DR. HELEN W.
 KAHLE, MR. AND MRS. GEORGE A.

- KAHLER, MRS. ROBERT W.
KAMINER, DR. AND MRS. BENJAMIN
KARPLUS, MRS. ALAN K.
KARUSH, DR. AND MRS. FRED
KELLEHER, MR. AND MRS. PAUL R.
KENDALL, MR. AND MRS. RICHARD E.
KEOSIAN, MRS. JESSIE
KEOUGHAN, MISS PATRICIA
KETCHUM, MRS. PAUL
KIEN, MR. AND MRS. PIETER
KINNARD, MRS. L. RICHARD
KISSAM, MR. WILLIAM M.
KIVY, DR. AND MRS. PETER
KOHN, DR. AND MRS. HENRY I.
KOLLER, DR. LEWIS R.
KORGEN, DR. BEN J.
KUFFLER, MRS. STEPHEN W.
LAFFERTY, MISS NANCY
LARMON, MR. JAY
LASTER, DR. AND MRS. LEONARD
LAUFER, DR. AND MRS. HANS
LAVIGNE, MRS. RICHARD J.
LAWRENCE, MR. FREDERICK V.
LAWRENCE, MR. AND MRS. WILLIAM
LAZAROW, DR. PAUL
LEATHERBEE, MRS. JOHN H.
LEBLOND, MR. AND MRS. ARTHUR
LEESON, MR. AND MRS. A. DIX
LEHMAN, MISS ROBIN
LEMAN, MRS. LUCY B.
LENER, DR. AND MRS. SAMUEL
LEPROHON, MR. JOSEPH
LEVINE, MR. JOSEPH
LEVINE, DR. AND MRS. RACHMIEL
LEVY, MR. STEPHEN R.
LINDNER, MR. TIMOTHY P.
LITTLE, MRS. ELBERT
LIVINGSTONE, MR. AND MRS. ROBERT
LOEB, MRS. ROBERT F.
LOVELL, MR. AND MRS. HOLLIS R.
LOW, MISS DORIS
LOWE, DR. AND MRS. CHARLES W.
LOWENGARD, MRS. JOSEPH
MACKEY, MR. AND MRS. WILLIAM K.
MACLEISH, MRS. MARGARET
MACNARY, MR. AND MRS. B. GLENN
MACNICHOL, DR. AND MRS.
EDWARD F., JR.
MAHER, MISS ANNE CAMILLE
MAHLER, MRS. HENRY
MAHLER, MRS. SUZANNE
MANSWORTH, MISS MARIE
MARSH, DR. AND MRS. JULIAN
MARTYNA, MR. AND MRS. JOSEPH C.
MASON, MR. APPLETON
MASTROIANNI, DR. AND MRS. LUIGI, JR.
MATHER, MR. AND MRS. FRANK J., III.
MATHERLY, MR. AND MRS. WALTER
MATTHIESSEN, DR. AND MRS. G. C.
MCCOY, MRS. LOIS
MCCUSKER, MR. AND MRS. PAUL T.
MCELROY, MRS. NELLA W.
MCLWAIN, DR. SUSAN G.
MCLARDY, DR. TURNER
MEIGS, MR. AND MRS. ARTHUR
MEIGS, DR. AND MRS. J. WISTER
MELILLO, DR. AND MRS. JERRY M.
MELLON, RICHARD KING, TRUST
MELLON, MR. AND MRS. RICHARD P.
MENDELSON, DR. MARTIN
METZ, DR. AND MRS. CHARLES B.
MEYERS, MR. AND MRS. RICHARD
MILLER, DR. DANIEL A.
MILLER, MR. AND MRS. PAUL
MIXTER, MR. AND MRS. WILLIAM J., JR.
MIZELL, DR. AND MRS. MERLE
MONROY, MRS. ALBERTO
MONTGOMERY, DR. AND MRS.
CHARLES H.
MONTGOMERY, DR. AND MRS.
RAYMOND B.
MOOG, DR. FLORENCE
MOORE, DR. JOHN AND BETTY
MORGAN, MISS AMY
MORSE, MRS. CHARLES L., JR.
MORSE, DR. M. PATRICIA
MOUL, DR. AND MRS. EDWIN T.
MURRAY, DR. DAVID M.
MYLES-TOCHKO, DR. CHRISTINA J.
NACE, DR. AND MRS. PAUL
NACE, MR. PAUL F., JR.
NELSON, DR. AND MRS. LEONARD
NELSON, DR. PAMELA
NEWTON, MR. WILLIAM F.
NICKERSON, MR. AND MRS. FRANK L.
NORMAN, MR. AND MRS. ANDREW E.
NORMAN FOUNDATION
NORRIS, MR. AND MRS. BARRY
NORRIS, MR. AND MRS. JOHN A.
NORRIS, MR. WILLIAM
O'HERRON, MR. AND MRS. JONATHAN
OLSZOWKA, MISS JANICE S.
O'NEIL, MR. AND MRS. BARRY T.
O'RAND, MR. AND MRS. MICHAEL
ORTINS, MR. AND MRS. ARMAND
O'SULLIVAN, DR. RENEE BENNETT
PAPPAS, DR. AND MRS. GEORGE D.
PARK, MRS. FRANKLIN A.
PARK, MR. AND MRS. MALCOLM S.
PARMENTER, DR. CHARLES
PARMENTER, MISS CAROLYN L.
PELTZ, MR. AND MRS. WILLIAM L.

PENDERGAST, MRS. CLAUDIA
PENDLETON, DR. AND MRS. MURRAY E.
PENNINGTON, MISS ANNE H.
PERKINS, DR. AND MRS. COURTLAND D.
PERSON, DR. AND MRS. PHILIP
PETERSON, MR. AND MRS. E. GUNNAR
PETERSON, MR. AND MRS. E. JOEL
PETERSON, MR. RAYMOND W.
PETTY, MR. RICHARD F.
PETTY, MR. WILLIAM
PFEIFFER, MR. AND MRS. JOHN
PLOUGH, MR. AND MRS. GEORGE H.
POINTE, MR. ALBERT
POINTE, MR. CHARLES
POTHIER, DR. AND MRS. AUBREY
PORTER, DR. AND MRS. KEITH R.
PRESS, DRs. FRANK AND BILLIE
PROSKAUER, MR. RICHARD
PROSKAUER, MR. JOSEPH H.
PROSSER, DR. AND MRS. C. LADD
PSALEDAKIS, MR. NICHOLAS
PSYCHOYOS, DR. ALEXANDRE
PUTNAM, MR. ALLAN RAY
PUTNAM, MR. AND MRS. WILLIAM A., III
RAYMOND, DR. AND MRS. SAMUEL
REESE, MISS BONNIE
REINGOLD, MR. STEPHEN C.
REYNOLDS, DR. AND MRS. GEORGE
REYNOLDS, MR. ROBERT M.
REZNIKOFF, MRS. PAUL
RICCA, DR. AND MRS. RENATO A.
RIGHTER, MR. HAROLD
RIINA, MR. AND MRS. JOHN R.
ROBB, MRS. ALISON A.
ROBERTS, MISS JEAN
ROBERTSON, MRS. C. W.
ROBINSON, DR. DENIS M.
ROOT, MRS. WALTER S.
ROSENTHAL, MISS HILDE
ROSLANSKY, DRs. JOHN AND PRISCILLA
ROSS, DR. AND MRS. DONALD
ROSS, DR. ROBERT
ROSS, DR. VIRGINIA
ROTH, DR. AND MRS. STEPHEN
ROWE, MR. DON
ROWE, MR. AND MRS. WILLIAM S.
RUBIN, DR. JOSEPH
RUGH, MRS. ROBERTS
RYDER, MR. AND MRS. FRANCIS C.
SAGER, DR. RUTH
SALGUERO, MRS. CAROL G.
SARDINHA, MR. GEORGE H.
SAUNDERS, DR. AND MRS. JOHN W.
SAUNDERS, MRS. LAWRENCE
SAUNDERS, LAWRENCE, FUND
SAWYER, MR. AND MRS. JOHN E.

SAZ, MRS. RUTH L.
SCHLESINGER, DR. AND MRS. R. WALTER
SCOTT, DR. AND MRS. GEORGE T.
SCOTT, MR. AND MRS. NORMAN E.
SEARS, MR. CLAYTON C.
SEARS, MR. AND MRS. HAROLD B.
SEARS, MR. HAROLD H.
SEAVER, MR. GEORGE
SEGAL, DR. AND MRS. SHELDON J.
SENF, DR. AND MRS. ALFRED
SHAPIRO, MRS. HARRIET S.
SHAPLEY, DR. ROBERT
SHEMIN, DR. AND MRS. DAVID
SHEPRO, DR. AND MRS. DAVID
SIMMONS, MR. TIM
SINGER, MR. AND MRS. DANIEL M.
SMITH, DRs. FREDERICK E. AND
MARGUERITE A.
SMITH, MRS. HOMER P.
SMITH, MR. VAN DORN C.
SNYDER, MR. ROBERT M.
SOLOMON, DR. AND MRS. A. K.
SPECHT, MRS. HEINZ
SPIEGEL, DR. AND MRS. MELVIN
SPOTTE, MR. STEPHEN
STEELE, MRS. JOHN H.
STEIN, MR. RONALD
STEINBACH, MRS. H. BURR
STETSON, MRS. THOMAS J.
STETTEN, DR. AND MRS. DEWITT, JR.
STETTEN, DR. GAIL
STEWART, MR. AND MRS. PETER
STONE, MR. ANDREW G.
STREHLER, DR. AND MRS. BERNARD
STUNKARD, DR. HORACE
SUDDITH, MR. WILLIAM
SWANSON, DR. AND MRS. CARL P.
SWOPE, MRS. GERARD, JR.
SWOPE, MR. AND MRS. GERARD L.
SZENT-GYÖRGYI, DR. AND MRS. ANDREW
TABOR, MR. GEORGE H.
TAYLOR, MR. JAMES K.
TAYLOR, MRS. MARGERY G.
TAYLOR, DR. AND MRS. W. RANDOLPH
TIETJE, MR. AND MRS. EMIL D., JR.
TIMMINS, MRS. WILLIAM
TODD, MR. AND MRS. GORDON F.
TOLKAN, MR. AND MRS. NORMAN N.
TRAGER, MRS. WILLIAM
TRIGG, MR. AND MRS. D. THOMAS
TROLL, DR. AND MRS. WALTER
TUCKER, MISS RUTH
TULLY, MR. AND MRS. GORDON F.
ULBRICH, MRS. MARY STEINBACH
VALOIS, MR. AND MRS. JOHN
VAN BUREN, MRS. HAROLD

VAN HOLDE, MRS. KENSAL E.
 VEEDER, MRS. RONALD A.
 VINCENT, DR. WALTER S.
 WAGNER, MR. MARK
 WAKSMAN, DR. AND MRS. BYRON H.
 WARD, DR. ROBERT T.
 WARE, MR. AND MRS. J. LINDSAY
 WARREN, DR. HENRY B.
 WARREN, DR. AND MRS. LEONARD
 WATT, MR. AND MRS. JOHN B.
 WEEKS, MR. AND MRS. JOHN T.
 WEINSTEIN, MISS NANCY B.
 WEISBERG, MR. AND MRS. ALFRED M.
 WEISS, MR. AND MRS. MALCOLM
 WHEELER, DR. AND MRS. PAUL S.
 WHITEHEAD, MR. AND MRS. FRED
 WHITNEY, MR. AND MRS.
 GEOFFREY G., JR.

WICHTERMAN, DR. AND MRS. RALPH
 WICKERSHAM, MR. AND MRS.
 A. A. TILNEY
 WIESE, DR. CONRAD
 WILHELM, DR. HAZEL S.
 WILSON, MR. AND MRS. T. HASTINGS
 WINN, DR. WILLIAM M.
 WINSTEN, DR. JAY A.
 WITTING, MISS JOYCE
 WOLFINSOHN, MRS. WOLFE
 WOODWELL, DR. AND MRS. GEORGE M.
 YNTEMA, MRS. CHESTER L.
 YOUNG-WALLACE, MISS NINA L.
 ZINN, DR. DONALD J.
 ZIPF, DR. ELIZABETH
 ZWILLING, MRS. EDGAR

III. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

IV. ARTICLES OF AMENDMENT

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

“No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.

“Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.

“The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members.”

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of \$10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI

Secretary of the Commonwealth)

V. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 16, 1985)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration or its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Members of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote

of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least two years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of four years. Eligibility for re-election shall be in accordance with the content of Article VIII(F) as applied to corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII(B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as ex officio members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive

Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. Except as otherwise provided below, the Corporation shall, to the extent legally permissible, indemnify each person who is, or shall have been, a Trustee, director or officer of the Corporation or who is serving, or shall have served, at the request of the Corporation as a Trustee, director or officer of another organization in which the Corporation directly or indirectly has any interest, as a shareholder, creditor or otherwise, against all liabilities and expenses (including judgments, fines, penalties and reasonable attorneys' fees and all amounts paid, other than to the Corporation or such other organization, in compromise or settlement) imposed upon or incurred by any such person in connection with, or arising out of, the defense or disposition of any action, suit or other proceeding, whether civil or criminal, in which he or she may be a defendant or with which he or she may be threatened or otherwise involved, directly or indirectly, by reason of his or her being or having been such a Trustee, director or officer.

The Corporation shall provide no indemnification with respect to any matter as to which any such Trustee, director or officer shall be finally adjudicated in such action, suit or proceeding not to have acted in good faith in the reasonable belief that his or her action was in the best interests of the Corporation. The Corporation shall provide no indemnification with respect to any matter settled or compromised, pursuant to a consent decree or otherwise, unless such settlement or compromise shall have been approved as in the best interests of the Corporation, after notice that indemnification is involved, by (i) a disinterested majority of the Board of Trustees or of the Executive Committee or, (ii) a majority of the Corporation's Members.

Indemnification may include payment by the Corporation of expenses in defending a civil or criminal action or proceeding in advance of the final disposition of such action or proceeding upon receipt of an undertaking by the person indemnified to repay such payment if it is ultimately determined that such person is not entitled to indemnification under the provisions of this Article XVI, or under any applicable law.

As used in this Article, the terms "Trustee," "director" and "officer" include their respective heirs, executors, administrators and legal representatives, and an "interested" Trustee, director or officer is one against whom in such capacity the proceeding in question or another proceeding on the same or similar grounds is then pending.

To assure indemnification under this Article of all persons who are determined by the Corporation or otherwise to be or to have been "fiduciaries" of any employee benefit plan of the Corporation which may exist from time to time, this Article shall be interpreted as follows: (i) "another organization" shall be deemed to include such an employee benefit plan, including, without limitation, any plan of the Corporation which is governed by the Act of Congress entitled "Employee Retirement Income Security Act of 1974," as amended from time to time ("ERISA"); (ii) "Trustee" shall be deemed to include any person requested by the Corporation to serve as such for an employee benefit plan where the performance by such person of his or her duties to the Corporation also imposes duties on, or otherwise involves services by, such person to the plan or participants or beneficiaries of the plan; (iii) "fines" shall be deemed to include any excise taxes assessed on a person with respect to an employee benefit plan pursuant to ERISA; and (iv) actions taken or omitted by a person with respect to an employee benefit plan in the performance of such person's duties for a purpose reasonably believed by such person to be in the interest of the participants and beneficiaries of the plan shall be deemed to be for a purpose which is in the best interests of the Corporation.

The right of indemnification provided in this Article shall not be exclusive of or affect any other rights to which any Trustee, director or officer may be entitled under any agreement, statute, vote of members or otherwise. The Corporation's obligation to provide indemnification under this Article shall be offset to the extent of any other source of indemnification or any otherwise applicable insurance coverage under a policy maintained by the Corporation or any other person. Nothing contained in this Article shall affect any rights to which employees and corporate personnel other than Trustees, directors or officers may be entitled by contract, by vote of the Board of Trustees or of the Executive Committee or otherwise.

VI. REPORT OF THE DIRECTOR

"The cook was a good cook, as cooks go; and as cooks go she went."

—Saki

Transition

It may be a violation of the best taste, but I believe that I should begin this report, my last from the Director's office of the MBL, on a personal note. I do so not because personal matters are worthy of the first position, but because a change of the Directorship of the MBL *is*. Since a good many Corporation Members have only the Annual Report in *The Biological Bulletin* as a source of comprehensive information on the Laboratory's work of the prior year, this seems to me the proper place and position for a statement on the change.

I wrote the Director's Report for 1985–6 in the Candle House. The window before which the computer monitor was placed overlooks an angle of Great Harbor and Vineyard Sound that I know as well as I know the shape of my hand. This report is being written in Charlottesville, Virginia, overlooking Thomas Jefferson's Academical Village, the heart of the University of Virginia. In June of 1986 I announced to the Executive Committee, and later that summer to the Trustees and the MBL community as a whole, that I would be leaving the Directorship and the Laboratory at the end of October, 1986. I had accepted, just prior to the first announcement, appointment in November as Vice President and Provost at the University of Virginia.

My effort was at the time, as it is now for readers not reached by the earlier one, to urge that this decision was made for reasons wholly positive as regards the MBL and my relationship with it—as of course it has been for Virginia. I found the Laboratory in—and no self-congratulation is intended—a much stronger position that might have been expected at the time of my coming to the Directorship. It now had enhanced administrative resources, vastly better physical facilities, a development and public relations program that was the envy of larger and richer institutions, heightened national and international recognition for the quality of its research and instructional programs, and a renewed loyalty of its Corporation membership.

I had never intended to remain a full-time (or better, double-time) administrator—for that is what, given the limits of my talents, the MBL job quickly became—beyond the end of a second term, which would have been in 1988. But I had no reason to be ashamed of what had been accomplished in nine years of tenure of the office. I would have stayed out the term and no more. But the offer from Virginia was a very attractive one, and it came from a University I had already served in other capacities and for whose intellectual life, Jeffersonian traditions, and physical place I have deep admiration. It had always been my intention to return to university life after fulfilling a deeply felt obligation to the MBL (where, in reality, I had *become* a biologist). In those circumstances the result of a cost/benefit analysis was clear: no great harm would be done to the Laboratory by my leaving it at that time, provided that a good transition of leadership could be managed and the Centennial planning could be kept on track; and the chances of another university place like as the one at Virginia appearing would diminish after 1988.

Thus it is that I am here and the MBL is there. I miss the Laboratory much more than I miss being its Director. There is every reason to believe that, given good health and some luck, opportunities to serve it in other ways will arise. I shall take advantage of them.

Key to the decision was a judgment as to the effectiveness with which a transition to new leadership could be made in 1986–7. Although I had as yet no way of identifying the eventual actors therein, my judgment was that the thing could and would be done. And so it proved. Fellow embryologist Richard Whittaker agreed generously to an infringement of his productive research in order to serve as acting Director. No better-qualified person could have been found for this role.

A distinguished search committee was quickly empaneled, fulfilled its responsibility for an exhaustive national search, and was able eventually to recruit as the MBL's next Director Harlyn O. Halvorson. His contributions to microbiology are recognized worldwide, and his activities at the MBL—as Trustee, Executive Committee Member, Course Director, and advisor to earlier Directors—comprise an unmatched record of dedicated and effective service.

The Centennial Committee, already in place at the time of my decision, was obviously off to a good start, and its programs for the Centennial year, which begins now (August, 1987), are a fine amalgam of high scientific standards, good taste, and potential outreach to a larger public.

I miss very much, in short, the view out my window from Candle House 301, but I feel justified in having had no fears for the health of the institution to which I had devoted so much physical and emotional energy in the vicinity of that view.

Management

Changing governance and management in an essentially academic institution such as the MBL is difficult in principle. It is made more difficult when there is a hundred-year history of outstanding achievement; and the difficulties multiply further when the organization is as idiosyncratic (or “horizontal,” as Chairman Gifford likes to describe it) as is ours. Change was and remains necessary, nevertheless, as I have urged in earlier Director's Reports and before what seem to me now numberless meetings of Trustees and committees. Nothing that has happened during the last, transition-preoccupied year has interfered—I am delighted to report—with the processes of orderly management change set in motion several years ago.

The Trustees—Board and Corporate—have been brought much more closely into touch with operations management and decision making than ever before. Indeed, this change has accelerated since the last Director's Report was published. The new Audit Committee, and other Trustees' committees functioning under revised charges, have rendered invaluable service to the Laboratory and its paid administration. As a consequence, our financial and political positions have been strengthened visibly, even since August of 1986. The Committee on Laboratory Goals, chaired conscientiously by Gerald Fischbach and charged a year ago, has produced a short but forceful report on those goals—on what they should be, and on what steps should be taken toward their accomplishment.

I support their recommendations with enthusiasm. This was an accomplished, critical, and independent committee: it is a great pleasure to note that had I written the report (to succeed my very solo effort of 1979), it might have been in a different prose style, but its content would have been indistinguishable from what is now before the MBL community for analysis, debate, and—action.

Treasurer David Currier, who served the Laboratory so handsomely in that position and whose banking skills made possible the splendid new MBL cottage development, retired with the well-earned thanks of the community and has been replaced by Robert Manz. Mr. Manz brings to the treasurer's responsibilities more than outstanding education, technical, and personal credentials: he is a former officer of Co-

pers and Lybrand, our external auditors for many years, and was a leading member of the team assigned to the MBL account. He knows the Laboratory as well as—perhaps better than—any full-time employee. The Treasurer's being another of those critical jobs for which the MBL must depend upon volunteers, *i.e.*, upon good will, we are doubly lucky to have the good will of Robert Manz.

Two new management positions were designed, funded, and filled in the course of the year. Richard D. Cutler became the Laboratory's first Facilities, Project, and Services Manager, and LouAnn D. King accepted appointment as Coordinator of Conferences and Housing. These attractive and experienced people have taken on challenging responsibilities subdivided and redefined from among the host of such carried by former General Manager Homer Smith.

Mr. Smith's retirement could certainly be described by the cliché, "The End Of An Era," and a pretty long era at that. But the description would fail. Homer has left his job, to be sure, but he has not left Woods Hole nor the MBL. There is no reason to believe that the "era" of his involvement with the Laboratory has ended; and we wish him and Cynthia Smith a retirement—if that word may be used—of continued good health and undiminished activity. That, I am sure, is the way they want it to be.

Donald Ayers, finally, now directs the fully functional Development Office, and we can be confident that his programs, aided and additionally monitored by Lisa Thimas (formerly Assistant to the Director), will flourish during the Centennial Year before us.

Remarkably, these are a mere sample, not the totality of appointments and change processes that took place during the transition year. Let no one be concerned about a let-down of effort or a diminution of those management skills of which the MBL has been so much in need. All that we need to be concerned about is the means of increasing further the rate of positive change.

Systems

In that connection it is noteworthy that three of the critical systems upon which those who must operate, and make decisions for, the MBL have been changed and improved during the year past. Most important, the many roadblocks, internal as well as external, impeding progress toward a rational system of overhead recovery have been eliminated. The system is now in operation, and although it works no better than those of our peer institutions, it is no worse. The high optimism implicit in such a negative statement will be understood by all who have grappled in decades past with the problem of reimbursing the Laboratory for its costs in housing and supporting research.

A job classification system was designed, discussed widely, and put in place for all MBL employees. This had been the majority wish for many years, and its fulfillment has indeed brought a measure of regularity and central accountability to the management of operations and personnel. The system includes not only a set of objective job descriptions and grades, but also processes for the hearing and adjudication of disputes and grievances. Already called upon for service in that connection has been the Classification Review Board established for the purpose and chaired, ably as always, by Joan Howard.

Last but not least, the Controller's department now has a greatly improved system of accounts and data management, aided by the appointment of an Assistant Controller with excellent accounting skills and a resident specialist in electronic data processing. It is not amiss to note that these changes are a direct consequence of the enhanced Trustee oversight initiated two years ago, to which I have referred above.

Research and education

Summer research activities and accomplishments were at their now accustomed high levels in the summer of 1986 and, as will be evident to readers of this report by the time it has been published, so will they be in 1987. There are very few laboratories or library accommodations unspoken for in summer at the MBL. The only concern I have heard expressed from time to time is that not enough applicants are rejected, or, to put it in the way it is usually phrased, that the MBL ought to have more of a choice among potential summer scientists. Perhaps so; but the financial realities and demography of those disciplines practiced by MBL summer investigators speak differently to me. I believe that we do very well to have a significant percentage of the most honored neuroscientists, cell biologists, developmental biologists, microscopists, and the like here at the MBL every summer, and it is insurance against stagnation that we can admit most of those others, not *yet* so honored perhaps, who have legitimate research to perform here. And lest the reader believe what is sometimes—unfairly and untruthfully—asserted, that the MBL is a club for its “regulars” and nothing more, let him inspect the roster of last summer’s MBL Fellows and independent investigators for the new and the still-young. The evidence is heartening. *No* other organization can boast so large and so diverse an assembly of biologists engaged upon serious research for a meaningful part of the year.

The year-round research program, focus of the plan presented by the Committee on Laboratory Goals, ended the year in about the same configuration as at the beginning. It was as large an enterprise as the MBL can accommodate within existing buildings; it was well-funded; and it was steadily productive. As I have said elsewhere, there is no university for which such a group, collected together as a Biology Department, would not be a prize.

There are, however, changes imminent. The National Institute for Neurological and Communicative Diseases and Stroke has decided, for what are considered in Bethesda to be good reasons, on an eventual recall of at least the bulk of its on-location program at the MBL. It is possible that a part of this effort will remain for several years, but under the existing directives, most of the program will revert to permanent residence on the NIH campus, with summer research at the MBL. Balancing this, there have been selections from among excellent applicant investigators for year-round accommodation, so that the *size* of the resident research program will not change very much in the near term, nor will its very high quality overall.

If, however, the Corporation and Trustees elect to implement the recommendations of the Fischbach committee, there will be major change indeed, and it will have to be accompanied by new methods of funding and supporting year-round scientists, new programs, a net increase in size, and large additions to the inventory of research space and general-use facilities.

The instructional program, whose unique contribution to the scientific manpower of this nation has been recognized by astonishingly (for these times) generous support from government and private agencies, fared well in 1986 and will clearly continue thus in 1987. In no small part this is due to the far-sighted support of such private donors as the Markey Trust, the Pew Memorial Trust, the Grass Foundation, and the Klingenstein Fund. But success and a tough-minded maintenance of the highest standards of quality are also testimony to the very spirit of the MBL, inherited and still vigorous, as established in its teaching programs by the Founders. In no small measure the smooth operation of our complex instructional enterprise—much more complex, in important ways, than the mounting of courses in a university—has depended, and will probably continue to depend, upon three things: (1) the flow of financial support for *indirect as well as direct* costs from private donors; (2) the collab-

oration, especially via equipment loans, of the world's leading manufacturers of scientific instruments; and (3) wise, artful, and minimally intrusive management of the courses and their people, as exemplified in the work of Joan Howard and her ever-helpful staff in the Office of Sponsored Programs.

Successful a story as this is, it will not remain so without continuous effort. It was a principle of the last administration, as it was of the MBL's first, that the instructional program shares the first priority with research, and that the teaching is as much on the moving frontier of our science as is the research of any particular laboratory. The principle will continue, I hope, to be held. Thus far the signs are good. By way of example, I might cite an unusual and already noteworthy collaboration of the MBL and the University of Georgia in the teaching of Plant Cell and Molecular Biology. And if the current plans for the next version of Embryology are implemented, it will be a further signal to the effect that curricular and organizational adventurousness need not be absent from teaching by an eminent faculty, to the world's most able classes of young biologists.

If all the above has the scent of hyperbole, I apologize but also deny any such purpose. The MBL is a very remarkable organism: the bare facts upon which this summary is based are printed in the accompanying pages of the Annual Report and in other MBL publications. I have simply written a summary, with a few laudatory adjectives where they are clearly justified. We tend easily to forget, as intimates of quality, how special a thing it is in the world. We do not forget problems with the same celerity: those, like the crying of a baby, evolve a sound that cannot be ignored.

Albert Szent-Györgyi

Among the score of Nobelists associated with the Laboratory during the two or three decades past, none made such a mark upon the day-to-day life of the place as did Albert Szent-Györgyi, who died at a ripe old age during the year. Of course that mark was to some extent a consequence of Szent-Györgyi's year-round residence in Woods Hole and at the MBL, from a time when his Institute for Muscle Research was virtually the only active group during the non-summer months until a few years ago, when it was well-eclipsed, in size and in visibility, by other year-round laboratories. But his influence did not depend solely upon presence. Prof's was a mind and a personality of most unusual strength. His intellectual power and personal charm were merely facets of the whole man, who was also possessed of moral power, a remarkable persuasiveness, an abundance of love and good cheer, and overlying all, an aesthetic imperative that drove him to the still-unexplored for its beauty first, and is utility second. During the height of his career, when I was a student here, he was for many of us the paradigm of the scientist as the spearhead of culture. Throughout his long life, even at the very end, when he and I could converse only in shouts, he was for me and, I know, for many others, the very model of a man.

Coda

The MBL nurtures us all, as men and women, as extenders of culture, as investigators, as teachers of the best science to the best students. Long may it flourish: and to it a happy hundredth birthday!

VII. REPORT OF THE TREASURER

This is my first report to you as Treasurer. Although I have been at the job for almost a year now, this is my first chance to say that I am privileged and honored to have an opportunity to contribute to this excellent and exciting institution. I look

forward to working with the staff, Executive Committee, Trustees, and Corporation as we move into the Laboratory's Second Century.

The financial statements of the Laboratory for the year ended December 31, 1986, follow this report. Before I comment on the financial results for the year, I must discuss some changes in presentation that have been made to the financial statements to reflect actions taken by the Corporation and the Executive Committee in the last year and to more clearly present the financial position of the Laboratory.

In the Statement of Support, Revenues, Expenses, and Changes in Fund Balances (Statement of Support), a separate column has been provided for the newly established Housing Enterprises Fund within the Current Unrestricted Fund. The Balance of Operations for this fund of \$112,294 has been transferred to a Reserve for Repairs and Replacements restricted to expenditure on housing. This is a major step forward for the Laboratory in providing for its financial future; I applaud the wisdom of your decision to establish this fund.

The financial statements no longer show the assets or results of operations of the Retirement Funds. These funds are not actually assets of the MBL but are owned by the Laboratory's pension plan, so they should not appear in the Laboratory's financial statements but in those of the Plan. To date there have been no separately issued financial statements of the Retirement Plan because of its size. The Plan has now grown large enough that provisions of the Employees' Retirement Income Security Act require separate financial statements of the Plan; these have been prepared, and the Retirement Fund has been removed from the Laboratory's financial statements.

Also affecting pension accounting, the Laboratory has adopted the new requirements of the Financial Accounting Standards Board on accounting for pension expense of the Laboratory (*i.e.*, its obligation to contribute to the plan). The new requirements seek to more accurately reflect the impact of changes in investment and annuity market conditions as well as employment and actuarial expectations of the employer's pension obligation. For the Laboratory this has meant a reduction in pension expense from \$142,833 in 1985 to \$82,682 in 1986, chiefly because of the favorable performance of the Retirement Plan's investment portfolio.

The Balance Sheet now reflects the market value of the Laboratory's invested funds, rather than their book value. I have recommended this change so that you will always have before you the actual market value of the endowment rather than a book number which reflects only the timing of past donations and the results of past investment activity.

I trust that these comments will assist you as you examine the Laboratory's Financial statements.

The results of operations for 1986 show a picture of continued current operating strength, with some fluctuations that bear watching. They suggest some significant improvements in the long-term strength of the Laboratory—if trends begun this year can be sustained and expanded—and they point to an agenda for future actions.

Total support and revenues increased from 1985 to 1986 by \$272,488 while expenses increased by \$269,648.

Within support and revenues, gains in gifts, recovery in indirect costs from the summer program (laboratory fees), and dormitory and dining fees were offset by declines in direct support for year-round research and the associated recovery of indirect costs, and slight declines in the income of Research Services and Marine Resources. It is encouraging to see some strength in fee income—it suggests that the Laboratory renders a measurable service which can be directly supported by the users of that service—but for the same reason it is discouraging to see the declines in Research Services and Marine Resources income. I have recommended to the Director that a

careful examination of all the fee for service income of the Laboratory be made in order to make the best possible match between service and income, and to guarantee the future economic viability of our services.

Gifts increased by more than \$200,000 in 1986. We have continued to receive strong support from the Pew and Markey foundations for the instructional program; we made significant strides in the Mellon match gift for Library endowment; and we received a \$200,000 gift from the Monsanto Corporation in anticipation of our Centennial celebration. Such dedicated support allows your laboratory to maintain the excellence of its programs. As your Treasurer, I must point out, however, that we are perilously dependent on the generosity of donors of gifts for current use. In order to achieve the assurance of continued excellent programs, we need to seek a dramatic increase in our endowment.

Within the expense categories, I call your attention to the increases in housing and depreciation expense, that in aggregate, amount to almost \$250,000. Of this amount, approximately \$133,000 is attributable to the additional housing units at Memorial Circle and an additional \$76,000 was expensed as administrative costs, thus completing our planned "full costing" of the separate Housing Enterprises Fund. More significantly, if you look down the column entitled "Housing Enterprises Fund," you will note that for the first time, the MBL, as projected and previously mentioned, has funded \$112,294 in depreciation costs associated with the housing enterprise. These funds are being set aside to help finance major future capital improvements in the housing facilities.

We again ended the year with an excess of revenues over expenses in the current unrestricted fund (\$70,590). You should thank your Controller, John Speer, for his role in achieving this result. The Laboratory is well served by John's vigilance over the operating budget and his ability to see clouds on the financial horizon and recommend a course long before the storm strikes.

As your Treasurer, I will never use the word "surplus" in connection with an excess of revenues over expenses until the Laboratory has been able to use that excess to fund depreciation on its plant. As I told the Trustees last winter and hope to demonstrate to the rest of the Corporation, the annual "surpluses" of the Laboratory are wiped out when depreciation is taken into account. We have in fact been able to maintain the quality of our physical plant through the generosity of our donors, but this means that the heroic capital fund raising efforts of our directors in the last ten years have been required to maintain rather than to improve the quality of our plant.

I believe we must set as a goal the funding of a significant portion of our depreciation expense from operating revenues. This year we took a modest first step towards that goal and transferred \$33,650 from the current unrestricted fund to a reserve for replacements. We must do much more in the future.

In this report I have tried to indicate those aspects of our financial condition that merit our attention and will require concerted action. I have no doubt left some of your questions on the financial performance of 1986 unanswered here, but I welcome them directly and will do my best to respond.

Coopers
& Lybrand

certified public accountants

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Boston, Mass. 02109

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telephone (617) 574-5000
TWX 710-321-0489
telex 6817018

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1986 and the related statement of support, revenues, expenses and changes in fund balances for the year then ended. Our examination was made in accordance with generally accepted auditing standards and, accordingly, included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported upon the financial statements of the Laboratory for the year ended December 31, 1985, which condensed statements are presented for comparative purposes only.

In our opinion, the financial statements referred to above present fairly the financial position of Marine Biological Laboratory at December 31, 1986 and its support, revenues, expenses and changes in fund balances for the year then ended, in conformity with generally accepted accounting principles applied on a basis consistent with that of the preceding year, except for the changes, with which we concur, in the method of accounting for investments as described in Note C, the method of accounting for pension expense as described in Note E and the method of accounting for pension funds as described in Note J.

Very truly yours,

Coopers & Lybrand

Boston, Massachusetts
April 18, 1987

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1986 and 1985

<i>Assets</i>	<i>1986</i>	<i>1985</i>	<i>Liabilities and Fund Balances</i>	<i>1986</i>	<i>1985</i>
Cash and savings deposits	\$ 238,120	\$ 197,668	Accounts payable and accrued expenses	\$ 333,859	\$ 594,534
Money market securities (Note I)	50,000	225,000	Deferred income	117,282	124,932
Accounts receivable, net of allowance for uncollectible accounts	425,840	374,301	Construction accounts payable	—	58,496
Receivables due for costs incurred on grants and contracts	562,375	741,206	Current portion of long-term debt	73,001	100,000
Other assets	8,663	11,859	Total current liabilities	524,142	877,962
Total current assets	1,284,998	1,550,034	Mortgage and note payable (Note H)	1,249,182	—
Investments, at market (Notes C and I)	10,093,443	8,989,633	Current unrestricted fund balances:	55,890	44,586
Land, buildings and equipment (Notes D and H)	19,193,896	17,805,051	Current unrestricted funds	—	—
Less accumulated depreciation	(7,143,565)	(6,579,654)	Housing enterprise funds	55,890	44,586
	12,050,331	11,225,397		—	—
Total assets	\$23,428,772	\$21,765,064	Current restricted fund balances:	55,890	44,586
			Unexpended grants	201,556	180,905
			Unexpended gifts	457,699	436,145
			Unexpended income of endowment funds	86,137	31,619
				745,392	648,669
			Endowment fund balances (Note C):		
			Income for unrestricted purposes	2,658,967	2,399,077
			Income for restricted purposes	2,592,671	1,941,644
				5,251,638	4,340,721
			Quasi-endowment fund balances (Note C):		
			Unrestricted	826,455	1,072,186
			Restricted	3,835,526	3,639,700
				4,661,981	4,711,886
			Plant fund balances:		
			Unrestricted	10,770,932	11,084,571
			Repairs and replacement reserve	169,615	—
			Restricted	—	56,669
			Total liabilities and fund balances	10,940,547	11,141,240
				\$23,428,772	\$21,765,064

The accompanying notes are an integral part of the financial statements.

STATEMENT OF SUPPORT, REVENUES, EXPENSES, AND CHANGES IN FUND BALANCES
for the year ended December 31, 1986
(with comparative totals for 1985)

	Current Funds			Plant Funds							
	Current Unrestricted Fund	Housing Enterprises Fund	Total Current Unrestricted Fund	Endowment Funds		Quasi-endowment Funds		Unrestricted	Restricted	Repairs and Replacement Reserve	Total Plant Funds
				Unrestricted	Restricted	Unrestricted	Restricted				
Support and revenues:											
Grant reimbursement of direct costs:											
Instruction										\$ 37,913	\$ 37,913
Research										3,327,603	3,327,603
Recovery of indirect costs related to research and instruction:											
Summer program	\$ 525,924		\$ 525,924							525,924	468,262
Year-round program	1,699,208		1,699,208							1,699,208	1,722,521
Other	32,107		32,107							32,107	66,226
Instruction	358,814		358,814							358,814	208,820
Tuition										287,415	270,237
Support activities:											
Dormitory		\$679,378	679,378							679,378	581,515
Dining hall		441,564	441,564							441,564	396,952
Library		240,315	240,315							240,315	215,348
Biological Bulletin		172,676	172,676							172,676	156,245
Research services		390,460	390,460							390,460	415,830
Marine resources		158,539	158,539							158,539	173,860
Other		500	500							500	(3,483)
Investment income		385,253	385,253							521,823	497,944
Additions to fund										—	29,381
Gifts	125,416		125,416					\$ 200,750		1,899,099	1,662,818
Miscellaneous revenue	119,404		119,404						\$ 3,039	153,729	112,716
Total support and revenues	4,650,180	679,378	5,329,558					200,750	3,039	10,927,067	10,654,579
Expenses:											
Instruction										960,727	1,063,601
Research	42,420		42,420							3,960,219	4,048,145
Scholarships and stipends										194,397	299,793
Support activities:											
Dormitory		491,551	491,551							491,551	382,655
Dining hall		439,994	439,994							439,994	388,712
Library		528,103	528,103							528,103	480,881
Biological Bulletin		150,551	150,551							150,551	153,593
Research services		772,553	772,553							772,553	720,509

[illegible]

The accompanying notes are an integral part of the financial statements.

NOTES TO FINANCIAL STATEMENTS

A. Purpose of the Laboratory:

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and nature history.

*B. Significant Accounting Policies:**Basis of Presentation—Fund Accounting*

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with specified activities or objectives.

Externally restricted funds may only be utilized in accordance with the purposes established by the donor or grantor of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when received and as related costs are incurred. Unrestricted current funds are reported as revenue when earned.

Endowment funds are subject to restrictions requiring that the principal be invested with income available for use for restricted or unrestricted purposes by the Laboratory. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds; however, the principal of these funds may be expended for various restricted and unrestricted purposes.

Fixed Assets

Fixed assets are recorded at cost. Depreciation is computed using the straight-line method over estimated useful lives of fixed assets.

Reclassifications

The financial statements for 1986 reflect certain changes in classification of revenue, expenses and changes in fund balances. Similar reclassifications have been made to amounts previously reported in order to provide consistency of the financial statements. In addition, the financial statements reflect in 1986 the segregation of the current unrestricted fund balance into two components: the current unrestricted and the housing enterprise fund balances.

Contracts and Grants

Revenues associated with contracts and grants are recognized in the statement of support, revenues, expenses and changes in fund balances when received and as related costs are incurred. The Laboratory reimbursement of indirect costs relating to government contracts and grants is based on negotiated indirect cost rates with adjustments for actual indirect costs in future years. Any over- or under-recovery of indirect costs is recognized through future adjustments of indirect cost rates.

Investments

Investments purchased by the Laboratory are carried at market value (Note C). Money market securities are carried at cost which approximates market value. Investments donated to the Laboratory are carried at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the average cost method. The Laboratory is the beneficiary of certain endowment investments which are held in trust by others. These investments are reflected in the financial statements. Every ten years the Laboratory's status as beneficiary is reviewed to determine that the Laboratory's use of these funds is in accordance with the intent of the funds.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of their proportionate share at market value adjusted for any additions or disposals to pooled funds.

C. Change in Accounting Method for Investments:

Effective January 1, 1986, the Laboratory adopted the accounting policy of reporting investments and the related fund balances at market value to more clearly reflect the financial impact of the Laboratory's investment policies. Investments and the related fund balances in prior years were reported at cost. The cumulative increase in the fund balances at December 31, 1986 and 1985 is as follows:

<i>Current Restricted Funds:</i>	<i>1986</i>	<i>1985</i>
Unexpended gifts		\$ 11,782
Endowment funds:		
Unrestricted	\$ 80,070	329,166
Restricted	81,780	215,763
Quasi-endowment funds:		
Unrestricted	31,438	126,333
Restricted	<u>142,527</u>	<u>436,913</u>
Increase in unrealized appreciation and related fund balances	<u>\$335,815</u>	<u>\$1,119,957</u>

This change has been retroactively applied to the fund balances as of the beginning of the year ended December 31, 1985 as follows:

Unexpended gifts	\$ (364)
Endowment funds:	
Unrestricted	263,969
Restricted	<u>311,892</u>
Quasi-endowment funds:	
Unrestricted	(3,747)
Restricted	<u>104,458</u>
Cumulative unrealized gain/loss	<u>\$ 676,208</u>

D. Land, Buildings, and Equipment:

The following is a summary of the unrestricted plant fund assets:

	<i>1986</i>	<i>1985</i>
Land	\$ 689,660	\$ 689,660
Construction in progress	—	140,826
Buildings	16,333,358	14,861,244
Equipment	<u>2,170,878</u>	<u>2,113,321</u>
	19,193,896	17,805,051
Less accumulated depreciation	<u>(7,143,565)</u>	<u>(6,579,654)</u>
	<u>\$12,050,331</u>	<u>\$11,225,397</u>

E. Retirement Fund:

During 1986, the Laboratory elected early application of Statement of Financial Accounting Standard No. 87, "Employer's Accounting for Pensions." This Statement establishes standards of financial accounting and reporting for an employer that offers pension benefits to its employees and supercedes earlier standards. The early election reduced the actuarially determined pension expense from \$132,866 to \$82,682.

The Laboratory has a noncontributory defined benefit pension plan for substantially all employees. Contributions are intended to provide for benefits attributed to the service date, but also those expected to be earned in the future.

Actuarial present value of benefit obligations:

Accumulated benefit obligation including vested benefits of \$1,484,283	1,686,685
Projected benefit obligation	2,561,619
Plan assets at fair value	2,608,987
Projected benefit obligation less than plan assets	47,368
Unrecognized net (gain) or loss	186,054
Prior service cost not yet recognized in net periodic pension cost	—
Unrecognized net obligation at March 1, 1986	(316,104)
Prepaid pension cost (pension liability) recognized in the statement of financial position	\$ (82,682)
Net pension cost for fiscal year ending December 31, 1986:	
Service cost—benefits earned during the period	138,391
Interest cost on projected benefit obligation	142,381
Actual return on plan assets	(319,877)
Net amortization and deferral	121,787
Net periodic pension cost	\$ 82,682

The actuarial present value of the projected benefit obligation was determined using a discount rate of 7.3% and rates of increase in compensation levels of 6%. The expected long-term rate of return on assets was 8%.

In addition, the Laboratory participates in the defined contribution pension program of the Teachers Insurance and Annuity Association. Expenses amounted to \$106,535 in 1986 and \$95,858 in 1985.

F. Pledges and Grants:

As of December 31, 1986 and 1985, the following amounts remain to be received on gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	December 31, 1986		December 31, 1985	
	Unrestricted	Restricted	Unrestricted	Restricted
1987	\$20,000	\$615,027	\$10,000	\$550,240
1988	10,000	40,764	10,000	15,000
1989	—	5,764	—	—
	<u>\$30,000</u>	<u>\$661,555</u>	<u>\$20,000</u>	<u>\$565,240</u>

G. Interfund Borrowings:

Interfund balances at December 31 are as follows:

	1986	1985
Current Funds		
Due to plant funds	\$(169,615)	\$ (56,669)
Due to endowment funds	(115,909)	(156,622)
Due to restricted quasi-endowment funds	(200,750)	—
	<u>\$(486,274)</u>	<u>\$(213,291)</u>

H. Mortgages and Notes Payable:

The mortgage note payable with a term of 26 years is in the amount of \$1.3 million bearing interest based on the bank's prime rate plus three quarters percent (.75%) on a floating basis for the initial five year period with a floor of 7.50% and a ceiling of 13.00%. The interest rate at December 31, 1986 was 9.00%. The mortgage loan is collateralized by a first mortgage on the land and properties known as Memorial Circle, with recourse in the event of default limited to this land and property and the related revenue. Principal and interest payments of \$15,000 are due and payable monthly commencing January 19, 1987.

Other notes payable consist of the following:

Unsecured note with interest at 7.90% with monthly principal payments of \$221.20 plus interest	\$ 9,194
Unsecured note with interest at 6.90% with monthly principal payments of \$394.71 plus interest	12,990
	<u>\$22,184</u>

At December 31, 1986, these mortgages and notes payable had aggregate future annual principal payments as follows:

	<i>Amount</i>
1987	\$ 73,001
1988	78,654
1989	83,899
1990	86,492
1991	94,127
Thereafter	906,010
	<u>1,322,183</u>
Less current portion	73,001
	<u>\$1,249,182</u>

I. Investments:

The following is a summary of the cost and market value of investments at December 31, 1986 and 1985 and the related investment income and distribution of investment income for the years ended December 31, 1986 and 1985.

	Cost		Market		Investment Income	
	1986	1985	1986	1985	1986	1985
<i>Endowment and Quasi-endowment</i>						
U.S. Government securities	\$2,278,843	\$1,974,662	\$ 2,384,842	\$2,075,648	\$240,056	\$202,695
Corporate fixed income	947,287	897,145	933,749	909,179	85,871	68,202
Common stocks	4,069,893	3,699,334	6,101,973	5,369,264	178,837	178,664
Preferred stock	9,611	2,860	17,975	9,183	3,214	1,001
Money market securities	639,155	603,718	639,155	610,610	29,667	35,632
Real estate	15,749	15,749	15,749	15,749	—	—
Total	7,960,538	7,193,468	10,093,443	8,989,633	537,645	486,194
Less custodian fees			(51,646)	(27,741)	(51,646)	(27,741)
			485,999	458,453	485,999	458,453
<i>Unrestricted Current Fund</i>						
Money market securities	50,000	225,000	50,000	225,000	35,824	39,491
Total investments	\$8,010,538	\$7,418,468	\$10,143,443	\$9,214,633	\$521,823	\$497,944

J. Change in Reporting of Retirement Fund:

Effective January 1, 1986, the Laboratory eliminated the reporting of its Retirement Fund (pension plan) as a separate fund in its financial statements to conform with the reporting practices of other nonprofit organizations. This change has been retroactively applied as of the beginning of the year ended December 31, 1985, with the following reductions in reported amounts for:

	1986	1985	1984
Investments (at cost)	\$1,879,410	\$1,688,670	\$1,713,055
Due to/from other funds	(82,628)	(146,253)	(105,328)
Retirement fund balance	1,962,038	1,834,923	1,607,727

VIII. REPORT OF THE LIBRARIAN

Our serial titles are now all entered into the On-Line OCLC catalog which is based in Ohio. The number of requests for copies of articles in our periodical collection has doubled since this project was completed. Over 350 libraries and laboratories sent requests during 1986, and we now serve a larger scientific community than we did before our collection was included in this database.

In preparation for the Centennial we have prepared a computer program for the records in the Archives. Lists of all scientists, students, lecturers, employees, and others who have been at the MBL since 1888 will be placed in an archival database. This information will be valuable to science historians, our public information office, and the library reference staff. Most of this material will be entered by Ruth Davis and her volunteer staff. Photographs held in the Rare Books and Archives area are being cataloged. Negatives are being made of a number of the rare ones. Many of these photographs will be used in a Centennial book that is being planned for 1988.

We gave one-day tours of the Library in June to two groups of librarians. One was a group from the Boston meeting of the Special Librarians Association and the other was a group named the "Rte. 128 Librarians" from the Hi-Tech libraries in that area.

Binding increased this year since we picked up a number of volumes where one or two issues were missing. We bound these noting the "lacks" on the spine. Over 3000 volumes were sent to the binders during the winter months.

IX. EDUCATIONAL PROGRAMS

SUMMER

BIOLOGY OF PARASITISM

Course directors

ENGLUND, PAUL, Johns Hopkins University
SHER, ALAN, NIAID/NIH

Other faculty, staff, and lecturers

ALDRITT, SUSAN, Harvard University
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BLOOM, BARRY, Albert Einstein College of Medicine
BROWN, KIM, University of Iowa
BURAKOFF, STEVEN, J., Dana-Farber Cancer Institute
BURNS, JAMES, Hahnemann University
BUTTERWORTH, ANTHONY, University of Cambridge, UK
CANTOR, CHARLES, Columbia University
CARTER, RICHARD, NIAID/NIH
CERAMI, ANTHONY, Rockefeller University
CLEVELAND, DON, Johns Hopkins University
DINTZIS, HOWARD, Johns Hopkins University
DONELSON, JOHN, University of Iowa
DOOLITTLE, RUSSELL, University of California, San Diego
DVORAK, JAMES, NIAID/NIH
GEARHART, PATRICIA, Johns Hopkins University
GOTTLIEB, MICHAEL, Johns Hopkins University
HART, GERALD W., Johns Hopkins University
HERELD, DALE, Johns Hopkins University
HOWARD, JAMES, Wellcome Laboratories

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 STRAND, METTE, Johns Hopkins University
 SUPLICK, KATHY, Hahnemann University
 TURNER, MERVYN J., Merck Sharp and Dohme Research Laboratories
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 WANG, CHING C., University of California, San Francisco
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 WARD, SAMUEL, Carnegie Institute
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EMBRYOLOGY: A MODERN COURSE IN DEVELOPMENTAL BIOLOGY

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MARINE ECOLOGY

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MICROBIOLOGY: MOLECULAR ASPECTS OF CELLULAR DIVERSITY

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 ANDREWS, BRIAN, NINCDS/NIH
 ARMSTRONG, KATIE, Rice University
 BRETT, ROGER, SUNY, Stony Brook
 CLAUDIO, TONI, Yale University Medical School
 CONNER, JOHN, Bell Laboratories
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 MARGULIES, DAVID, Columbia University College of Physicians and Surgeons
 MATSUMOTO, STEVE, Harvard University
 MCNIVEN, MARK, University of Maryland
 MILLER, CHRISTOPHER, Brandeis University
 MOOSEKEE, MARK, Yale University
 MURRAY, JOHN, University of Pennsylvania
 PAULSEN, HENRY, Yale University Medical School
 RAVIOLA, ELIO, Harvard Medical School
 REESE, THOMAS S., NINCDS/NIH/Marine Biological Laboratory
 ROLE, LORNA, Columbia University
 ROSENBLUTH, JOHN, New York University
 ROWLAND, L., Columbia University
 SCHNAPP, BRUCE, Marine Biological Laboratory
 SHEETZ, MICHAEL, Washington University School of Medicine
 SILMAN, ISRAEL, Weizmann Institute of Science, Israel
 SPUDICH, JOHN, Albert Einstein College of Medicine
 STERNWEIS, PAUL, University of Texas Health Center
 WILLARD, ALAN L., University of North Carolina

Students

BLEY, KEITH R., Yale University
 CARLBERG, MATS, University of Lund, Sweden
 FEDEROFF, HOWARD J., Massachusetts General Hospital
 FERNANDEZ-VALLE, CRISTINA, University of Miami
 HALPERN, MARNIE E., Yale University
 HERRIGAN, FRANK T., Stanford University
 LEW, DANIEL J., The Rockefeller University
 PLEASURE, SAMUEL J., University of Pennsylvania
 PORTER, DEVRA, Vanderbilt University
 SCHWEIZER, FELIX E., Universitat Basel, Switzerland
 SUPATTAPONE, SURACHAI, Johns Hopkins School of Medicine
 ZUMBROICH, THOMAS J., University of Oxford, Oxford, UK

PHYSIOLOGY

Course director

GOLDMAN, ROBERT, Northwestern University

Other faculty, staff, and lecturers

ALBRECHT-BUEHLER, G., Northwestern University
 BECKERLE, MARY, University of North Carolina
 BENDER, WELCOME, Harvard University
 BLOOM, KERRY, University of North Carolina
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 DESSEV, GEORGE N., Northwestern University
 FILETI, LISA, Boston University
 FUKUI, YOSHIO, Osaka University, Japan
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 HYAMS, JEREMY, University College, UK
 JONES, JONATHAN, Northwestern University
 KIEHART, DAN, Harvard University
 LEINWAND, LESLIE, Albert Einstein College of Medicine
 LINDBERG, UNO, University of Stockholm, Sweden
 MAYRAND, SANDRA, Worcester Foundation for Experimental Biology
 MATTOX, ANDREW, Marine Biological Laboratory
 McNALLY, ELIZABETH, Albert Einstein College of Medicine
 MORELAND, ROBERT, Dana Farber Cancer Institute
 OLMSTED, JOANNA, University of Rochester
 PARYSEK, LINDA, Northwestern University Medical School
 PEDERSON, THORU, Worcester Foundation for Experimental Biology
 PTASHNE, MARK, Harvard University
 RICH, ALEXANDER, Massachusetts Institute of Technology
 RUDERMAN, JOAN, Duke University
 RUSHFORTH, ALICE, Earlham College
 RUSKIN, BARBARA, Harvard University
 SCHWARTZ, LAWRENCE M., University of North Carolina
 SHAPIRO, LUCY, Albert Einstein College of Medicine
 SINGER, REBECCA, Albert Einstein College of Medicine
 SOHN, REGINA LEE, Albert Einstein College of Medicine
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 WHITMAN, GEORGE, Worcester Foundation for Experimental Biology
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 YEH, ELAINE, CIBA-GEIGY

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 BEEMAN, ANNE M., Dartmouth Medical School
 BISWAS, SURAJIT K., University of Pennsylvania
 BLACK, KRISTIN, University of California, Berkeley
 BRADLEY, DAVID, University of Pennsylvania
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 DASSO, MARY C., Cambridge University, England, UK
 DEYST, KATHERINE A., Tufts University
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 KENNEY, LINDA J., University of Pennsylvania
 LAUERMAN, TOD W., Johns Hopkins University
 MEINHOF, CARL-GHORG, University of California, San Diego
 MELUH, PAMELA B., University of Maryland
 MOHL, VIRGINIA K., Washington State University
 REGINATO, ANTONIO M., University of Pennsylvania
 RUDOLPH, KAREN M., Dartmouth College
 SARDET, CLAUDE C. S., Centre National de la Recherche Scientifique, France
 SEGRE, GINO V., Massachusetts General Hospital/Harvard Medical School
 SYMONS, MARC H. C., Weizmann Institute of Science, Israel
 THALER, CATHERINE D., University of California, Riverside
 TROXELL, CYNTHIA L., University of Colorado
 WADSWORTH, WILLIAM G., University of Missouri
 WATSON, CORNELIUS A., Wesleyan University
 YANAGIHARA, RICHARD, National Institute of Neurological and Communicative Disorders and Strokes/NIH
 YORK, KAREN PICKWICK, University of Pennsylvania
 ZAND, MARTIN S., Northwestern University Medical School

SPRING

ANALYTICAL AND QUANTITATIVE LIGHT MICROSCOPY IN BIOLOGY, MEDICINE, AND MATERIALS SCIENCE

April 3-10, 1986

Course director

INOUE, SHINYA, Marine Biological Laboratory

Other faculty, staff, and lecturers

AKINS, ROBERT, University of Pennsylvania
 ELLIS, GORDON W., University of Pennsylvania
 KNUDSON, ROBERT A., Marine Biological Laboratory
 LANNI, FREDERICK, Carnegie Mellon University
 LUBY-PHELPS, KATHERINE, Carnegie Mellon University
 LUTZ, DOUGLAS, Harvard University
 SALMON, EDWARD A., University of North Carolina
 TAYLOR, D. LANSING, Carnegie Mellon University
 WALKER, RICHARD, University of North Carolina

Commercial faculty

ABROMOWITZ, MORTIMER, Olympus Corporation of America
 ALEXANDER, SCOTT, Nikon, Inc.
 BEACH, DAN, Carl Zeiss, Inc.
 BREEN, BILL, Interactive Video Systems
 DEMIAN, JEFFREY, Nikon, Inc.
 ESSER, HERMAN, Ikegami Electronics (USA), Inc.
 FOSTER, BARBARA, Carl Zeiss, Inc.
 GRACE, JOHN, Crimson Camera Technical Sales, Inc.
 HANNAWAY, WYNDHAM, G. W. Hannaway Associates
 HINSCH, JAN, E. Leitz, Inc.
 JONES, JEFFREY, Olympus Corporation of America
 KELLER, ERNST, Carl Zeiss, Inc.
 KIMURA, T., Olympus Corporation of America

KLEIFGEN, GERRY, Dage-MIT
 KNUTRUD, PAUL, Interactive Video Systems
 ORWELL, PATTY, E. Leitz, Inc.
 PACKARD, MEL, Quantex Corporation
 PRESLEY, PHIL, Carl Zeiss, Inc.
 REGAN, ANN, Ikegami Electronics (USA), Inc.
 RUBINOW, JERRY, Universal Imaging Corporation
 SCHEIRER, KURT, Nikon Inc.
 SCOTT, ERIC, Ikegami Electronics (USA), Inc.
 TAYLOR, RICHARD, Colorado Video
 THOMAS, PAUL, Dage-MIT
 VRATNEY, MELANA, Nikon Inc.

Students

ANDERSON, DONALD M., Woods Hole Oceanographic Institution
 ARONSON, JOHN F., Wistar Institute
 BARI, DANIEL, Universidad Nacional de Cuyo-Conicet, Argentina
 CAVICCHIA, JUAN CARLOS, Universidad Nacional de Cuyo-Conicet, Argentina
 CHAMBERS, EDWARD L., University of Miami School of Medicine
 DUBINSKY, JANET M., Washington University School of Medicine
 FEIGENSON, GERALD W., Cornell University
 GALLAGER, SCOTT M., Woods Hole Oceanographic Institution
 GLANZMAN, DAVID L., Howard Hughes Medical Institute
 KHAN, SHAHID M. M., Albert Einstein College of Medicine
 KLEITMAN, NAOMI, Washington University School of Medicine
 MCCULLOH, DAVID H., University of Miami School of Medicine
 MILLER, PAUL, Bell Laboratories
 NORRIS, CAROLYN, Bardeen Labs
 PALAZZO, ROBERT E., University of Virginia
 REESE, TOM, Marine Biological Laboratory
 RUSSELL, JAMES T., NIH
 SCHULZE, ERIC, University of California, San Francisco
 SHEEHY, PAUL A., NIH
 VALE, RONALD D., Marine Biological Laboratory
 VOYVODIC, JAMES, Washington University School of Medicine
 WIGSTON, DONALD, Emory University School of Medicine
 WOMACK, MARY, Howard Hughes Medical Institute
 YEAGER, MARK D., Cornell University

SHORT COURSES

CELL AND MOLECULAR BIOLOGY OF PLANTS

August 4-14, 1986

Coordinators

DURE, LEON, The University of Georgia
 KEY, JOE L., The University of Georgia

Lecturers

AUSUBEL, FRED, Massachusetts General Hospital
 CHUA, NAM, Rockefeller University
 CLEGG, MICHAEL, University of California, Riverside
 CROUCH, MARTHA, Indiana University
 FRALEY, ROB, Monsanto Company

HEPLER, PETER K., University of Massachusetts
 LEVINGS, C. S. III, North Carolina State University
 MALMBERG, RUSSELL, The University of Georgia
 MEAGHER, RICHARD, The University of Georgia
 PALEVITZ, BARRY, The University of Georgia
 QUAIL, PETER, University of Wisconsin
 SOMMERVILLE, CHRIS, Michigan State University
 STROMMER, JUDITH, The University of Georgia
 TIMBERLAKE, BILL, University of California, Davis
 VARNER, JOE, Washington University
 YODER, OLIN, Cornell University

Students

AGARWAL, MUNNA LAI, Centre National de la Recherche Agronomique, France
 ARMOUR, SUSAN, CIBA-GEIGY Corp.
 ARMOUR, TOBY, Edgartown, Massachusetts
 BECK, JAMES J., CIBA-GEIGY Corp.
 CAROZZI, NADINE, CIBA-GEIGY Corp.
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 CURRY, L. JEANNE, University of Massachusetts
 ELLIOTT, WILLIAM, Hartwick College
 GOLDMAN, PEG, New Haven, Connecticut
 HANLEY, SUSAN, BioTechnical International Inc.
 HAUGE, BRIAN, Massachusetts General Hospital
 LOTSTEIN, RICHARD, CIBA-GEIGY Corp.
 MCCABE, BRIAN, Bloomington, Indiana
 MINEO, LORRAINE, Lafayette College
 MULCARE, DONALD J., Southeastern Massachusetts University
 NAM, HONG GIL, Massachusetts General Hospital
 NOBLE, REGINALD D., Bowling Green State University
 ROSE, VIRGINIA, Concord, Massachusetts
 SAXENA, INDER MOHAN, University of Texas, Austin
 WILLIAMS, SHERICCA C., CIBA-GEIGY Corp.

BASIC IMMUNOCYTOCHEMICAL TECHNIQUES IN TISSUE SECTIONS AND WHOLE MOUNTS

October 19–25, 1986

Course directors

BELTZ, BARBARA, Harvard Medical School
 BURD, GAIL D., University of Arizona

Course assistants

KENT, CARLA, University of Arizona
 KOBIESKI, LINDA, Harvard Medical School

Students

AKANA, SUSAN FONG, University of California, San Francisco
 BRADFUEHRER, PETER D., Cornell University
 FOERSTER, ANNE, McMaster University, Canada
 GETCHELL, MARILYN L., Wayne State University
 HAMILTON, KATHRYN A., New England Medical Center
 HAMMAR, KATHERINE M., Marine Biological Laboratory/NIH
 HELLUY, SIMONE, The University of Alberta, Canada

KRUSZEWSKA, BARBARA, University of Texas, Austin
 LAUFER, HANS, University of Connecticut
 ROSS, LINDA S., University of Texas, Austin
 SASAVAGE, NANCY L., Bethesda Research Laboratories
 TILSON, HUGH A., National Institute of Environmental Health Sciences
 WHITE, JOEL, The Florida State University
 WOOD, SUSAN F., Marine Biological Laboratory/BUMP
 ZIGMOND, RICHARD E., Harvard Medical School

X. RESEARCH AND TRAINING PROGRAMS

SUMMER

PRINCIPAL INVESTIGATORS

ALLEN, NINA S., Wake Forest University
 ALLEN, LABORATORY, Dartmouth College
 ANDERSON, WINSTON A., Hunter College
 ARMSTRONG, CLAY M., University of Pennsylvania
 ARMSTRONG, PETER B., University of California, Davis
 ATWOOD, KIM, Marine Biological Laboratory
 AUGUSTINE, GEORGE, University of Southern California
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 BARLOW, ROBERT B., Syracuse University
 BARRY, M., Albert Einstein College of Medicine
 BARRY, SUSAN R., University of Michigan
 BEAUGE, LUIS ALBERTO, Instituto de Investigacion Medica, Argentina
 BEGENISICH, TED, University of Rochester Medical Center
 BENNETT, MICHAEL V. L., Albert Einstein College of Medicine
 BEZANILLA, FRANCISCO, University of California, Los Angeles
 BLUNDON, JAY A., University of Maryland
 BODZNICK, DAVID, Wesleyan University
 BORGESE, THOMAS A., Lehman College
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 BROWN, JOEL E., Washington University
 BURDICK, CAROLYN J., Brooklyn College
 BURGER, MAX M., University of Basel, Switzerland
 CARMELIET, PETER, University of Leuven, Belgium
 CHANG, DONALD C., Baylor College of Medicine
 CHAPPELL, RICHARD L., Hunter College
 CHARLTON, MILTON P., University of Toronto, Canada
 CLEVELAND, MARK V. B., Braintree Laboratories
 COHEN, LAWRENCE B., Yale University
 COHEN, WILLIAM D., Hunter College
 CONDOURIS, GEORGE A., New Jersey Medical School
 COOPERSTEIN, SHERWIN J., University of Connecticut
 CORNWALL, M. CARTER, Boston University School of Medicine
 D'AVANZO, CHARLENE, Hampshire College
 DEWEER, PAUL, Washington University
 DUBAS, FRANÇOISE, Whitney Laboratory of Marine Biology
 DUBE, FRANÇOIS, University of Quebec
 DUNLAP, KATHLEEN, Tufts University School of Medicine

ECKBERG, WILLIAM R., Howard University
EHRlich, BARBARA, Albert Einstein College
FEINMAN, RICHARD, SUNY Health Sciences Center
FESTOFF, BARRY W., University of Kansas Medical Center
FISHMAN, HARVEY M., University of Texas Medical Branch
FREADMAN, MARVIN, Marine Biological Laboratory
FULKERSON, JOHN PRYOR, University of Connecticut School of Medicine
GADSBY, DAVID C., Rockefeller University
GAINER, HAROLD, NICHD/NIH
GARBER, SARAH S., Brandeis University
GEORGE, EDWIN B., Case Western Reserve University
GILBERT, DANIEL L., NINCDS/NIH
GIUDITTA, ANTONIO, University of Naples, Italy
GOULD, ROBERT, New York Institute for Basic Research
GOVIND, C. K., University of Toronto
GRAF, WERNER M., Rockefeller University
HALVORSON, HARLYN O., Brandeis University
HEPLER, PETER K., University of Massachusetts
HIGHSTEIN, STEPHEN M., Washington University
HILL, ROBERT B., University of Rhode Island
HILL, SUSAN DOUGLAS, Michigan State University
HOSKIN, FRANCIS C. G., Illinois Institute of Technology
HORN, RICHARD, University of California Medical School
HUMPHREYS, TOM, University of Hawaii
JOHNSON, KENNETH A., Pennsylvania State University
JOSEPHSON, ROBERT K., University of California, Irvine
KALTENBACH, JANE C., Mount Holyoke College
KAMINER, BENJAMIN, Boston University
KAO, PETER, Columbia University
KAPLAN, EHUD, Rockefeller University
KEYNAN, ALEXANDER, Memorial Sloan Kettering Cancer Center
KEM, WILLIAM R., University of Florida
KORNBERG, HANS, University of Cambridge, UK
LANDOWNE, DAVID, University of Miami
LANGFORD, GEORGE M., University of North Carolina
LASEK, RAYMOND J., Case Western Reserve University
LAUFER, HANS, University of Connecticut
LEVIS, RICHARD A., Rush Medical Center
LINDGREN, CLARK, Duke University Medical Center
LIPICKY, RAYMOND JOHN, Food and Drug Administration
LISMAN, JOHN, Brandeis University
LLINAS, RUDOLFO R., New York University
LOEWENSTEIN, WERNER R., University of Miami
MALBON, CRAIG C., State University of New York, Stony Brook
MATTESON, DONALD R., University of Pennsylvania
METUZALS, J., University of Ottawa, Canada
MORRELL, FRANK, Rush-Presbyterian-St. Luke's Medical Center
MORRELL, LEYLA DE TOLEDO, Rush-Presbyterian-St. Luke's Medical Center
MULLINS, LORIN J., University of Maryland
NARAHASHI, TOSHIO, Northwestern University
NELSON, LEONARD, Medical College of Ohio
NOE, BRYAN D., Emory University
NOLEN, THOMAS G., Yale University
OHKI, SHINPEI, State University of New York, Buffalo
PALEVITZ, BARRY A., University of Georgia

PARSONS, THOMAS D., University of Pennsylvania School of Dental Medicine
PALMER, JOHN D., University of Massachusetts, Amherst
PEROZO, EDUARDO, Instituto Venezolano de Investigaciones Cientificas, Venezuela
PIERSON, BEVERLY K., University of Puget Sound
POOLE, THOMAS J., Upstate Medical Center
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PURVES, DALE, Washington University
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REID, JOHN, Hampshire College
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REYNOLDS, GEORGE T., Princeton University
RICKLES, FREDERICK R., University of Connecticut Health Center
RIPPS, HARRIS, University of Illinois College of Medicine
ROME, LAWRENCE C., University of Tennessee
ROSS, WILLIAM, New York Medical College
RONAN, MARK, Wesleyan University
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RUSSELL, JOHN M., University of Texas
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SEGAL, SHELDON J., Rockefeller Foundation
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SMITH, STEPHEN J., Yale University School of Medicine
SPECK, WILLIAM T., Rainbow Babies & Childrens Hospital
SPIEGEL, EVELYN, Dartmouth College
SPIEGEL, MELVIN, Dartmouth College
STANLEY, ELIS F., NINCDS/NIH
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TAKEDA, KENNETH, Universite Louis Pasteur, France
TASHIRO, JAY SHIRO, Kenyon College
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WEIDNER, EARL, Louisiana State University
WEISS, DIETER G., Institute of Zoology, FRG
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ZIGMAN, SEYMOUR, University of Rochester School of Medicine

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BARRETT, DENNIS, University of Denver
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COHEN, SEYMOUR S., MBL
COLEN, B. D., *Newsday*
D'ALESSIO, GIUSEPPE, University of Naples
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DIPEOLU, OLUSEGUN O., Tuskegee University
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FELDMAN, SUSAN, New Jersey Medical School
FIELD, GEORGE, Center for Astrophysics
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HERSKOVITS, THEODORE T., Fordham University

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ILAN, JUDITH, Case Western Reserve University
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LAZAROW, PAUL B., Rockefeller University
LEE, JOHN J., City College of CUNY
LEIGHTON, JOSEPH, The Medical College of Pennsylvania
LEVITZ, MORTIMER, NYU Medical Center
LLOYD, DAN, Simmons College
LONG, CAROLE A., Hahnemann University School of Medicine
LORAND, LASZLO, Northwestern University
LYNCH, ELIZABETH, Kenyon College
MACKENZIE, DEBORA OLIVIA, *New Scientist*
MACLEISH, WILLIAM H., Houghton-Mifflin
MAIENSCHIN, JANE, Arizona State University
MARFEY, ANNE, Danish Writers Guild
MARINE RESEARCH, INC.
MASER, MORTON, Woods Hole Educational Associates
MATSUMURA, FUMIO, Michigan State University
MAUTNER, HENRY G., Tufts University School of Medicine
MAUZERALL, DAVID, Rockefeller University
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McCOY, FLOYD W., Lamont-Doherty Geological Observatory
MELE, SUZANNAH, Kenyon College
MILLER, JULIE ANN, *Science News*
MILLER, MELISSA, Kenyon College
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MITCHELL, RALPH, Harvard University
MIZELL, MERLE, Tulane University
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MOORE, JOHN W., Duke University Medical Center
MORSE, PATRICIA M., Northeastern University
MUSACCHAI, X. J., University of Louisville
NAGEL, RONALD L., Albert Einstein College of Medicine
NICKERSON, PETER A., SUNY, Buffalo
OLINS, ADA L., University of Tennessee, Oak Ridge
OLINS, DONALD E., University of Tennessee, Oak Ridge
OLSZOWKA, ALBERT J., SUNY, Buffalo
OTT, KAREN, University of Evansville
PEISACH, JACK, Albert Einstein College of Medicine
PERSON, PHILIP, VA Medical Center, Brooklyn, New York
POOLE, ALAN F., MBL
PRICE, BILL, Kenyon College

PROVASOLI, LUIGI, Yale University
RUSCH, ROBERT, Gonzaga University
RAEBURN, PAUL, Associated Press
REINER, JOHN M., Albany Medical College
RINGER, STEPHEN, Childrens Hospital
ROBINSON, DENIS, MBL
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RUSSELL-HUNTER, W. D., Syracuse University
SCHLESINGER, R. WALTER, University of Medicine and Dentistry of New Jersey
SCHMIDT, SUSANNE, Cape Cod Planning and Economic Development
SEAVER, GEORGE, Seaver Assoc.
SHAPLEY, ROBERT, Rockefeller University
SHEMIN, DAVID, Northwestern University
SHEPARD, FRANK, Woods Hole Data Base
SHEPRO, DAVID, Boston University
SHRIFTMAN, MOLLIE STARR, North Nassau Mental Health Center
SLUDER, GREENFIELD, Worcester Foundation for Experimental Biology
SOHN, JOEL, Joel Sohn Seafood
SPECTOR, ABRAHAM, Columbia University
SPOTTE, STEPHEN, Mystic Marinelife Aquarium
STEINBERG, MALCOLM S., Princeton University
STEPHENS, MICHAEL J., Rutgers University
STEPHENSON, WILLIAM K., Earlham College
STEVENS, CHARLES F., Yale Medical School
SZENT-GYÖRGYI, ANDREW G., Brandeis University
SZENTKIRALYI-SZENT-GYÖRGYI, EVA M., Brandeis University
TONE, JEFFERSON, Kenyon College
TRAGER, WILLIAM, Rockefeller University
TUTTLE, FRANK, Kenyon College
TWEEDLE, KENYON S., University of Notre Dame
VAN HOLDE, K. E., Oregon State University
WAGNER, ROBERT R., University of Virginia
WAINIO, WALTER, Rutgers University
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WARREN, LEONARD, Wistar Institute
WEBB, H. MARGUERITE, MBL
WEINER, JONATHAN, Doylestown, Pennsylvania
WHEELER, GEORGE E., Brooklyn College
WHITTENBERG, BEATRICE, Albert Einstein College of Medicine
WHITTENBERG, JONATHAN, Albert Einstein College of Medicine
WICHTERMAN, RALPH, MBL
WILBUR, CHARLES G., Colorado State University
WOLKEN, JEROME J., Carnegie Mellon University
WORGUL, BASIL V., College of Physicians and Surgeons, Columbia University
YATHIRAJ, SANJAY, Kenyon College
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 YANDOW, TIMOTHY

Trainees

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 RUDNICK, DAVID, University of Rhode Island

Visiting scientists

JORDAN, MARILYN J.
 O'BRIEN, W. JOHN
 RUBLEE, PARKE
 WARING, RICHARD

XI. HONORS

FRIDAY EVENING LECTURES

SIMBERLOFF, DANIEL, Florida State University, 27 June, "*Academic Ecology and Environmental Problems: Red Scales, Vampire Bats, and Spotted Owls*"
 ALBERSHEIM, PETER, University of Georgia, 4 July, "*Oligosaccharins—A New Class of Regulatory Molecules in Plants and Animals*"
 BROWN, DONALD D., Carnegie Institution of Washington, 11 July, "*The Molecular Basis of Differential Gene Expression*"
 STEVENS, CHARLES F., Yale University School of Medicine, 17, 18 July, Forbes Lectures, "*Molecular Basis for the Brain's Electrical Activity: I. Electrical Excitability of Neurons; II. Communication between Neurons*"
 KAISER, DALE, Stanford University School of Medicine, 25 July, "*Cell-Cell Interactions in a Simple Developmental Pathway*"
 REESE, THOMAS S., NINCDS, NIH, and Marine Biological Laboratory, 1 August, "*Kinesin—An MBL Project*"
 DOOLITTLE, RUSSELL F., University of California, San Diego, 8 August, "*Evolution of the Vertebrate Plasma Proteins*"
 KANDEL, ERIC R., College of Physicians & Surgeons of Columbia University and the Howard Hughes Medical Institute, 15 August, Lang Lecture, "*The Long and Short of Memory*"
 SELA, MICHAEL, The Weizmann Institute of Science, 22 August, "*From Synthetic Antigens to Synthetic Vaccines*"
 TRINKAUS, J. P., Yale University, 29 August, "*Metazoan Cell Movements: Invasion and Morphogenesis*"

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 KOENIG, GERD, MPI fur Entwicklungsbiologie, FRG
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Albert Einstein College of Medicine	Boston University Medical School
American Health Foundation	Bowling Green State University
Amherst College	Bowman Gray Medical School
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PHYSIOLOGICAL ROLES OF PROSTAGLANDINS AND OTHER EICOSANOIDS IN INVERTEBRATES

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ABSTRACT

Prostaglandins and other biologically active derivatives of polyunsaturated fatty acids have been detected in a large number of invertebrate species. A brief summary of the mammalian background of arachidonic acid metabolism is provided, and the physiological significance of these compounds in invertebrates is reviewed. Topics include regulation of ion flux, temperature regulation, reproductive biology, cell aggregation, and host-parasite interactions. Finally, perspectives on current and possible future research are offered.

INTRODUCTION

The term eicosanoid was introduced and used by Corey *et al.* (1980) to describe the various biologically active derivatives of eicosapolyenoic fatty acids, especially arachidonic acid. So far, we know of four major groups of eicosanoids: the prostaglandins (PGs), the hydroperoxy- and hydroxyeicosatetraenoic acids (HPETEs and HETEs), the leukotrienes (LTs), and the lipoxins (LXs). Interest in the significance of eicosanoids in the biology of mammals stems from physiological studies conducted in the early twentieth century. In the earliest reference to one group of eicosanoids, the PGs, Jappelli and Scafa (1906) noted that extracts of dog prostate glands caused paralysis of central respiratory control and changed heart rates when injected into dogs and rabbits. The discovery of PG pharmacological activity in human seminal fluids (Kurzrok and Lieb, 1930) probably marks the beginning of the detailed studies of the clinical significance of these compounds. Elucidation of the chemical structures of PGs in the early 1960's (Bergström *et al.*, 1962a, b) greatly increased the pace of research and discovery, hindered in that decade mainly by the limited availability of working quantities of purified compounds. It is now known that PGs are present and play important roles in almost all mammalian tissues and fluids (Horrobin, 1978). Examples of PG action include pathophysiological actions such as mediation of the inflammatory response (which we commonly block by ingestion of aspirin) and participation in the blood-clotting cascade, as well as physiological actions such as contraction of smooth muscle.

The growth of PG research began with initial physiological observations, along with isolation and structural determinations of individual PGs. This was followed by the development of techniques to produce PGs in a commercially profitable way for clinical and biological studies. Commercial production of PGs evolved from biosynthesis from appropriate precursor fatty acids using large-scale enzyme preparations, through the discovery of naturally occurring sources of PGs and of intermediates in chemical synthesis to economical total synthesis. Hence, the first report of PGs in

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Abbreviations: PG = prostaglandin, LT = leukotriene, HETE = hydroxyeicosatetraenoic acid, HPETE = hydroperoxyeicosatetraenoic acid, LX = lipoxin.

an invertebrate animal, the gorgonian coral *Plexaura homomella* (Weinheimer and Spraggins, 1969), met with tremendous interest, not as a zoological discovery, but as a commercial source of PG for laboratory study. In the years between this first discovery of a potentially economical source of PG and the development of appropriate synthetic strategies, the search for other biological sources of PGs turned up many examples of their occurrence in marine invertebrates, albeit at tissue concentrations far below the point of commercial interest.

One of the PGs in greatest abundance in the coral, 15-*epi*-PGA₂, is not pharmacologically active in the usual mammalian biological assays for PG activity (Nakano, 1969). Chemical modification of the naturally occurring form to clinically useful structures, as well as commercial, ecological, and environmental aspects of sustained PG yield from coral have been reviewed (Theoder, 1977; Berte, 1981; Bundy, 1985). Other papers describe evidence for the occurrence of PGs in over one hundred invertebrate species. Christ and van Dorp (1972) detected PG-biosynthesis activity in five invertebrates—including two coelenterates, a mollusc, an annelid and an arthropod—but not in two insect species. Using a classical bioassay for the pharmacological effect of PG on contraction of mammalian smooth muscle, Nomura and Ogata (1976) detected PGs in a procordate, and in representatives of Echinodermata, Mollusca, Annelida, Coelenterata, and Arthropoda (including an insect). PGs were also detected by bioassay in saliva of another terrestrial arthropod, the tick *Boophilus microphus* (Dickinson *et al.*, 1976; Higgs *et al.*, 1976). Using radioimmunoassay, Shemesh *et al.* (1979) found PGs in reproductive organs and salivary glands of another tick. Since all PGs are formed from a common intermediate, prostaglandin-endoperoxide, PG synthesis could be inferred from activity of prostaglandin-endoperoxide synthetase. Morse *et al.* (1978) detected this enzyme activity in 41 species of coelenterates collected in the Caribbean Sea and the Pacific Ocean. Gromov *et al.* (1982) used radioimmunoassay to estimate amounts of two PGs in a snail. Korotchenko *et al.* (1983) found smooth muscle-contracting activity in 10 echinoderm species; they also refer to finding PG activity in 40 other invertebrates.

Aside from detection of PGs in a large number of invertebrate species, certain reports suggest that eicosanoids play fundamental physiological roles in representatives of many invertebrate phyla. Such findings are interesting because they provide insights into the details of regulatory physiology. Interest extends to an evolutionary axis because discovery of eicosanoid physiology especially in the very early phyla suggests that the significance of these compounds is not limited to vertebrate and clinical physiology, but was established early in metazoan evolution.

Evolutionary interest may eventually extend to plants, as well. Gregson *et al.* (1979) described the occurrence of two PGs in the red alga *Gracilaria lichenoides*, and Janistyn (1982) reported chemical identification of PGF_{2α} in the flowering plant *Kalanchoe blossfeldiana*. A prostaglandin-like compound was produced from linolenic acid by an enzyme preparation of flaxseed (Zimmerman and Feng, 1978). The physiological significance of these compounds in plants is not clear, but compounds that inhibit PG-biosynthesis in mammals inhibited growth in four fungus species (Herman and Herman, 1985; Kerwin *et al.*, 1986). Earlier inhibitor studies showed inhibition of flowering in *Pharbitis nil* (Groenewald and Visser, 1974). Although these findings are preliminary, they suggest that eicosanoids may be of broad biological significance.

The goal of this review is to provide an appreciation of the physiological significance of eicosanoids in invertebrate animals. Since the appropriate nomenclature and physiological background comes from decades of work on various mammal systems, it is useful to begin with a background from mammal studies.

A BACKGROUND FROM MAMMALIAN STUDIES

Upon stimulation by various agonists, many mammal cells hydrolyze polyunsaturated fatty acids (PUFAs), by action of phospholipase A₂, from the beta carbon of membrane phospholipids. Three C20 PUFAs—dihomo- γ -linolenic (C20:3n6), arachidonic (C20:4n6), and eicosapentaenoic (C20:5n3) acids—may be metabolized by one of two major pathways into biologically active molecules. In the cyclooxygenase pathway, PUFAs are transformed into prostaglandins and thromboxanes, whereas the lipoxygenase pathway leads to hydroperoxy- and hydroxypolyenoic fatty acids which are themselves biologically active as well as further metabolized into lipoxins and leukotrienes. Since these are all derivatives of C20 PUFAs, they may be collectively referred to as eicosanoids. The following description of the biosynthesis and physiological roles of these compounds in mammals is assembled from several reviews and books (Horrobin, 1978; Samuelsson *et al.*, 1978; Hansson *et al.*, 1983; Samuelsson, 1983; Serhan *et al.*, 1985), and is presented with minimum referencing.

PGs are C20 carboxylic acids with a five-membered ring variously substituted at C9 and C11, and two aliphatic chains featuring a substitution at C15 and one, two, or three double bonds. The structures of the principle PGs are shown in Figure 1. PGs are designated as lettered and numbered series. The numbers indicate the number of aliphatic double bonds, giving rise to the one-, two-, and three-series PGs. The letters are associated with the particular pattern of substitutions on the five-membered ring: PGE features C9 keto, C11 hydroxyl substitutions; PGF a C9,C11 dihydroxyl pattern; PGD a C9 hydroxyl, C11 keto arrangement. PGs of the A, B, D, E, and F series are so distinguished.

Biosynthesis of PGs is a multistep operation beginning with formation of the prostaglandin endoperoxides—first PGG—by action of microsomal prostaglandin endoperoxide synthetase. The same enzyme also cleaves the hydroperoxy group of PGG to form PGH. PGH is the root intermediate in the synthesis of the primary PGs: PGD is formed by a glutathione-S-transferase, PGE requires prostaglandin endoperoxide E isomerase and PGF prostaglandin endoperoxide reductase; PGI is formed by prostaglandin endoperoxide I isomerase and thromboxane A (TxA) by prostaglandin endoperoxide thromboxane A isomerase.

PGs have been detected in most mammalian tissue systems where they are involved in many well-catalogued (Horrobin, 1978) physiological activities. Examples of PG action include contraction of smooth muscle (*i.e.*, uterine, gut, and blood vessel), attenuating cellular response to hormones, and release of digestive acid in the stomach. Thromboxane A₂ is a potent inducer of platelet aggregation; its name is taken from its origin, the thrombocytes.

Lipoxygenase pathways first lead to hydroperoxy fatty acids which can be reduced by peroxidases, and possibly by non-enzymatic reactions, to corresponding hydroxy fatty acids (Fig. 2). Arachidonic acid is the best-studied lipoxygenase substrate in mammals, and oxygen can be added at various positions, leading to 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acids (the various HETEs). Di- and tri-hydroxy fatty acids also can be formed by lipoxygenase acting on the same fatty acid substrate more than once; another route to trihydroxy acids is by way of an epoxy-hydroxy acid. While PGs are involved in various physiological as well as pathophysiological actions, the lipoxygenase products apparently are involved in pathophysiological actions such as bronchial constriction. The lipoxygenase reactions are found in defense systems such as the various leukocytes, macrophages, monocytes, lung, and spleen. HETEs are biologically active in defense roles. For example, 5-, 9-, and 11-HETE are all active in inducing the chemokinesis and chemotaxis associated with migration of eosinophils into the site of certain hypersensitivity reactions.

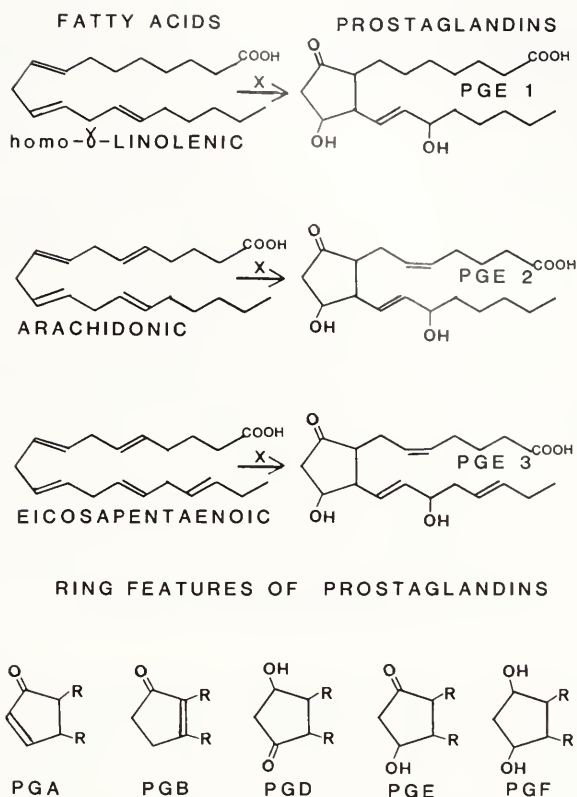


FIGURE 1. Relationship between the 1-, 2-, and 3-series prostaglandins and their parental polyunsaturated fatty acids, respectively C20:3n6, C20:4n6, and C20:5n3, is indicated by the arrows. $\times$ indicates cyclooxygenase activity. Ring features of five prostaglandins are shown in the lower panel where R stands for the aliphatic chains shown on the complete structures.

The leukotrienes (LTs; Fig. 3) were discovered during work on rabbit polymorphonuclear leukocytes, and take their names from this and the conjugated triene structure they have in common. The following description of LTs comes from the review by Samuelsson (1983). There are two classes of leukotrienes: the cysteine-containing group (LTC₄, LTD₄, and LTE₄), and LTB₄, which is not substituted. Biosynthesis of the LTs begins with formation of 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) by action of lipoxygenase followed by conversion to LTA₄ by abstraction of a hydrogen and elimination of a hydroxyl anion, catalyzed by a soluble enzyme, dehydrase. LTA₄ is converted to LTB₄ by hydrolase, or into the parental cysteine-containing LT (LTC₄) by a glutathione-S-transferase. The cysteine-containing LTs feature a thioether linkage at C6 to cysteine; LTC₄ is γ -glutamyl-cysteinyl-glycyl substituted; glutamyl transpeptidase elimination of the glutamine residue forms cysteinylglycyl LTD₄ which can be metabolized into cysteinyl LTE₄.

LTs have been identified in several cell systems including rabbit, human, mouse, and rat leukocytes; mouse and rat macrophages; and human and guinea pig lung. The biological significance of these compounds lies in their identification as the slow-reacting substance of anaphylaxis (SRS-A). This material is a mediator in asthma and other mammalian hypersensitivity reactions; SRS-A is released with other mediators

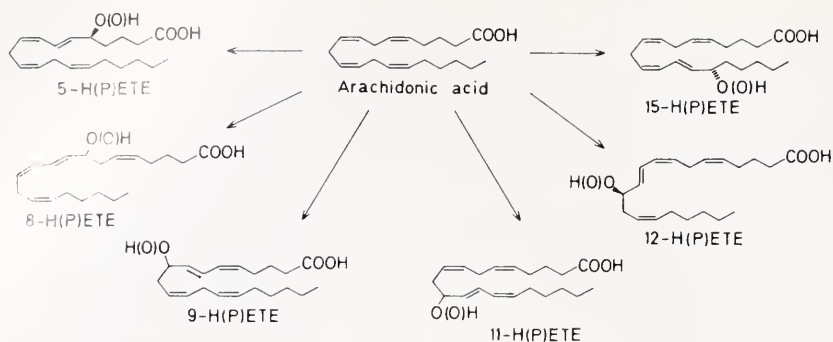


FIGURE 2. Structures of lipoxygenase metabolites of arachidonic acid, hydroxyeicosatetraenoic and hydroperoxyeicosatetraenoic acids.

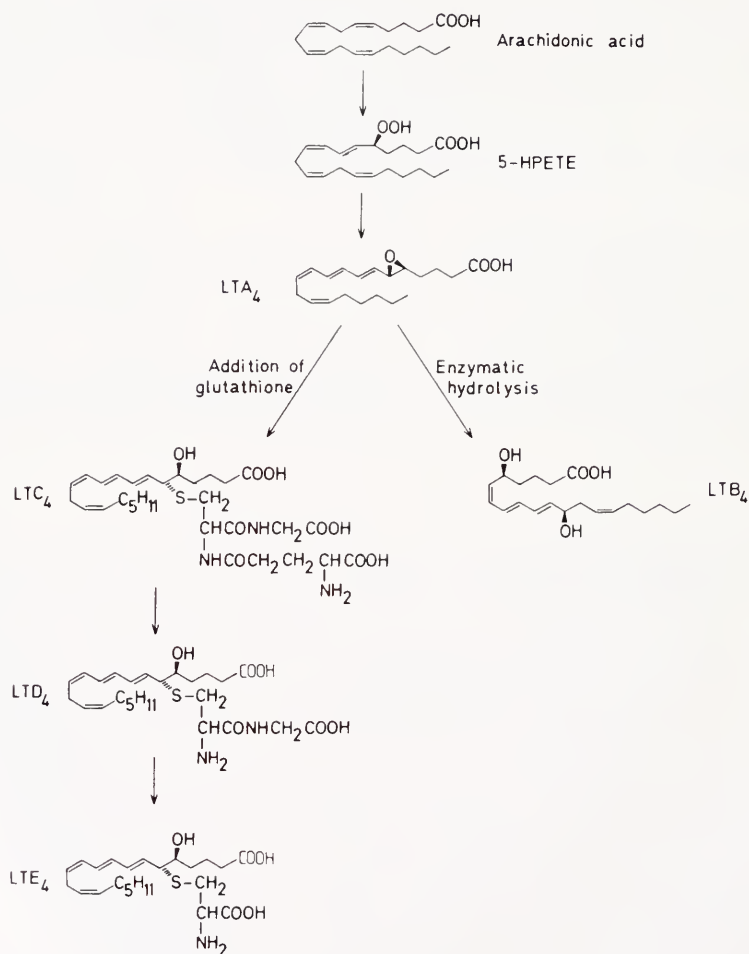


FIGURE 3. Structures of leukotrienes.

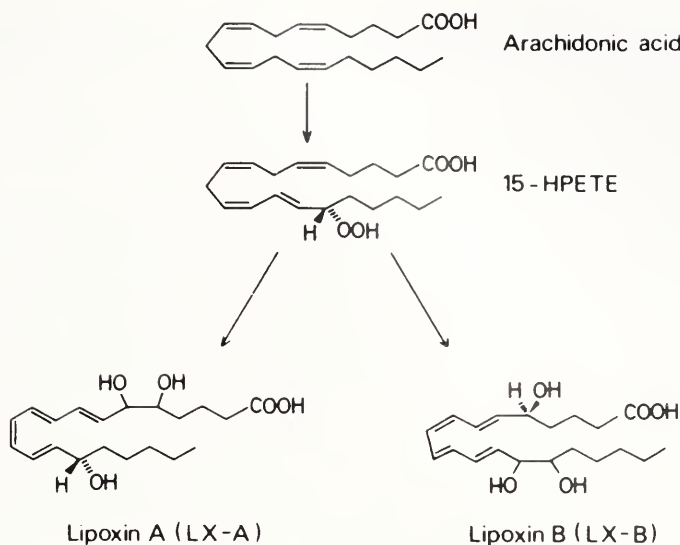


FIGURE 4. Structures of lipoxins.

after interaction of antigens such as pollen with immunoglobulin. SRS-A is a mixture of the cysteine-containing LTs. LTB_4 , which does not contain cysteine, stimulates enzyme release, adhesion of neutrophils to endothelial cells, and movement of fluids through vessel walls in microcirculation. Lindgren *et al.* (1985) showed that LTs occur in the rat brain—most prominently in the hypothalamus and median eminence—and that they may be involved in hormone release by brain cells.

The lipoxins (Lx; Fig. 4) are the most recently discovered metabolites of arachidonic acid. They share the characteristic feature of a conjugated tetraene structure. Two major LXs, LXA and LXB, were formed by human leukocytes; LXA stimulated oxygen metabolism and generation of active oxygen species in human neutrophils. The action of LXA in neutrophils differs from the action of leukotriene B_4 and may represent another physiological mechanism of host defense. Lipoxins appear to be formed by 5-lipoxygenase activity on a substrate formed by 15-lipoxygenase metabolism of arachidonic acid. (The trivial name lipoxins is an abbreviation of lipoxygenase interaction products.)

The PGs, LTs, and LXs are involved in basic physiological processes at the cellular level and appear to be especially important in various pathophysiological responses such as inflammation, blood-clotting, asthma, and tumor growth. Due to their clinical significance, much effort is directed toward appreciating the regulation of arachidonic acid metabolism and developing specific inhibitors of PG, LT, and LX biosynthesis. Specific compounds will be mentioned in the contexts of biological studies in various invertebrate systems. Here it should be mentioned that within a given mammalian system there is considerable tissue variation in the effects of various inhibitors; moreover, there is variation between mammalian species. In light of tissue and specific variations in cyclooxygenase and lipoxygenase systems in mammals, one notes that the considerable literature on mammals should not be taken as a set of rules of the biochemistry of fatty acids in invertebrates. It is more appropriate to interpret the background as a loose set of guidelines, likely to be misleading at

crucial points in our consideration of the physiological significance of eicosanoids in invertebrates.

PHYSIOLOGICAL SIGNIFICANCE OF EICOSANOIDS IN INVERTEBRATES

Regulation of ion flux

Like other freshwater bivalves, *Ligumia subrostrata* maintains its body fluids hyperosmotic to the aquatic medium, largely by regulating the flux of sodium, its major blood cation (Dietz, 1977, 1979). PGE_2 appears to be a component of the sodium regulation system because inhibition of endogenous PG-biosynthesis by injection of indomethacin, a potent cyclooxygenase inhibitor in mammals, increased sodium flux. The effect lasted about 15 hours, approximately doubling the control values. Alternatively, when PGE_2 was injected in parallel experiments, sodium influx declined about 5-fold from control values. Since chloride concentrations and sodium outflux remained unchanged during these experiments, Graves and Dietz (1979) concluded that PGE_2 participates in ion regulation by specifically controlling sodium influx. A tissue specificity may also exist because indomethacin modified the activity of the epithelial cells involved in sodium uptake without changing urinary sodium loss.

Indomethacin stimulated sodium influx in a dose-dependent way over the concentration range of 0.05 to 0.25 $\mu\text{mol/g}$ dry wt. Other PG-synthetase inhibitors in mammals—meclofenamate (a cyclooxygenase inhibitor), and dexamethasone (which inhibits phospholipase A_2 , and hence, regulates substrate availability)—also stimulate sodium uptake (Saintsing and Dietz, 1983). The stimulatory effect of PG-synthetase inhibitors was neutralized by co-injection of PGE_2 , supporting the view that PG is part of the system regulating epithelial sodium flux. PGE_2 reduces influx; reduction of PGE_2 biosynthesis may increase influx by attenuating the PG inhibition of uptake, but positive stimulation seems to depend on a biogenic amine, 5-hydroxytryptamine (5-HT, or serotonin), rather than on another PG since $\text{PGF}_{2\alpha}$ acts much like PGE_2 (Saintsing and Dietz, 1983). Cyclic AMP (cAMP) also stimulates sodium uptake (Graves and Dietz, 1982), which suggests that PG inhibition and 5-HT stimulation of sodium flux may both function via antagonistic effects on adenyl cyclase activity.

Arachidonic acid injections apparently increased renal outflux of sodium without changing epithelial uptake. Graves and Dietz (1979) suggested that the arachidonic acid may initially alter renal function, and be metabolized too quickly to allow formation of inhibitory levels of PGE_2 in epithelial tissue. Another possibility is that ion regulation is more complex (Graves and Dietz, 1982). If, as in mammals, arachidonic acid is potentially metabolized into a variety of prostanoid compounds, then we can imagine one metabolite, PGE_2 , inhibiting epithelial uptake while others, not yet identified, modify renal ion flux in ways still unknown.

The idea that PGs regulate epithelial sodium uptake in a freshwater mussel is based mostly on pharmacological treatments with appropriate compounds. Saintsing *et al.* (1983) showed the presence of PGs in *L. subrostrata* extracts by RIA, lending further support to natural occurrence and biological activity in an aquatic invertebrate.

PGE_2 is also involved in ion regulation in the marine bivalve *Modiolus demissus* (Freas and Grollman, 1980). When isolated gills were subjected to hypoosmotic stress by incubation for 60 minutes in 25% seawater, there was a 10-fold increase in PGE_2 released into the medium, suggesting an increase in biosynthesis and release of the

PG. In addition to this osmotic action on PG release, there is a specific ionic effect. To test for possible ionic effects, gills were incubated in artificial seawater of fixed osmotic concentration, but selectively free of sodium, calcium, potassium, or magnesium. Only the magnesium-free artificial seawater stimulated gills to increase PGE_2 release. However, the apparent osmotic effect is not due solely to depletion of environmental magnesium because gills incubated in hypoosmotic seawater with normal magnesium concentrations also induced increased PG release. Hence, gill tissues of this marine bivalve respond to changes in osmotic and ionic concentrations.

In mammals, the physiological activities of many PGs are mediated by specific cellular receptor sites. Freas and Grollman (1981) showed the existence of specific PGA_2 binding sites in homogenates of gills, mantles, siphons, adductor, and upper and lower visceral masses. In gills, these sites were ionic, pH dependent, and reversible. To date this is the only study of PG binding sites in invertebrate tissues; such a finding adds considerable verisimilitude to physiological properties of PGs.

Mediation of behavioral thermoregulation and fever

PGE_1 appears to mediate febrile response to infection in a number of mammals, including monkeys (Crawshaw and Still, 1975), sheep (Hales *et al.*, 1973), rabbits (Stitt, 1973; Lin, 1978), cats (Milton and Wendlandt, 1970; 1971), and guinea pigs (Szekely and Komaroni, 1978). Fever also occurs in non-mammalian vertebrates, although the increased body temperatures appear to be mediated by behavioral as opposed to endogenous physiological mechanisms. Behavioral fever has been observed in frogs (Casterlin and Reynolds, 1977a, Myhre *et al.*, 1977), a lizard (Bernheim and Kluger, 1976), and several fishes (Reynolds *et al.*, 1976).

Some aquatic invertebrates express behavioral fever in response to bacterial infection by moving into a zone of warmer water. The freshwater crayfish *Cambarus bartoni* exhibited a 2°C behavioral fever after inoculation with a suspension of killed bacteria (*Aeromonas hydrophila*) by choosing higher temperatures in a gradient trough (Casterlin and Reynolds, 1977b). This behavioral response to infection may be mediated by endogenous formation of PGE_1 because increasing doses of the PG also induced 1 to 3.5°C fevers when injected over the range of 50 to 500 μg /individual (Casterlin and Reynolds, 1978). Three marine arthropods—the American lobster *Homarus americanus*, the pink shrimp *Penaeus duorarum*, and the horseshoe crab *Limulus polyphemus*—similarly increased their temperature preference by more than 4°C in response to 100 μg injections of PGE_1 (Casterlin and Reynolds, 1979).

Two terrestrial arthropods, the scorpions *Buthus occitanus* and *Androctonus australis*, regulated their body temperatures by selecting appropriate positions along temperature gradients in a sand box. *A. australis* increased temperature preferences by 15°C and *B. occitanus* by 20°C after treatment with physiological doses of PGE_1 (Cabanac and Le Guelte, 1980). Although it is not known whether these species generate fever due to bacterial infection, it appears that PGs may be involved in some aspect of behavioral thermoregulation.

Together, these reports suggest that PGs may be some part of the thermoregulatory physiology of many invertebrates. The idea is based on the observation of increased body temperatures in response to individual doses of a single compound, namely PGE_1 . Important detailed biochemical questions remain unanswered: do PGs naturally occur in these species? Does PG biosynthesis increase after infection, but before the febrile response? Do all PGs induce fever, or is a more specific set of these compounds involved? Research in this area may assume ecological interest, as suggested by remarks below.

Among terrestrial invertebrates, many medium to large size insects regulate thoracic temperatures to a set point suited to the high metabolic demands of powered flight by behavioral (Casey, 1981) or physiological (Kammer, 1981) means. In addition to flying insects, thermoregulation has been studied in caterpillars of two sphinx moths, *Hyles lineata* and *Manduca sexta* (Casey, 1976, 1977). *H. lineata* appears to sustain high body temperatures and correspondingly high rates of feeding by basking in appropriate postures; *M. sexta* does not maintain high temperatures even though feeding and growth rates are reduced considerably at cooler temperatures. These different behaviors appear to be linked to differences in predator defense mechanisms and in seasonal availability of their host plants. Other caterpillars, including the butterflies *Vanessa io* and *V. urtica*, huddle in groups, resulting in increased body temperature and development rates (Mosebach-Pukowski, 1938). Similarly, the larvae of wax moths thermoregulate, partly, by huddling or scattering (Smith, 1941).

Many insect species are resistant to viral infection when maintained at higher temperatures (Tanada, 1967). Watanabe and Tanada (1972) reviewed several lepidopteran cases of insect viruses which do not cause lethal infections at higher temperatures, including larvae of the armyworm *Pseudaletia unipunctata*, the cabbage looper *Trichoplusia ni*, and the corn ear worm *Heliothis zea*. Hence, behavioral thermoregulation in invertebrates may effect such biological parameters as feeding and development rates, and resistance to disease. PGs may be an important biochemical mediator in this area of physiological ecology.

Control of hatching

In the barnacle *Balanus balanoides*, full egg-laying involves passing eggs along oviducts into ovisacs produced by oviducal glands. Fully formed egg masses are finally released into the mantle cavity, where they remain until hatching which corresponds with spring algal blooms (Crisp, 1962; Clare *et al.*, 1985). The synchrony of spring bloom and egg hatching could be related to a component in the nutrition of adult barnacles. However, Crisp and Spenser (1958) showed that seawater extracts of unfed and fed adults were equally effective in inducing hatching. They proposed a barnacle hatching substance, endogenously produced by adults, and showed that the substance acts upon the musculature of mature embryos, not on the egg case.

The hatching substance appeared to be a PG (Clare *et al.*, 1982, 1985). The substance is extractable in a system optimized for PGs, it behaves like a PG on thin layer chromatography, and extracts of the dried cortex of a commercial source of PG (the gorgonian *Plexura homomalla*) acted biologically and chemically like barnacle hatching substance. Extracts made in the presence of aspirin—a PG-synthetase inhibitor in mammals—did not induce hatching. Clare *et al.* (1985) concluded that barnacle hatching substance is either a PG or a PG-like compound.

Subsequent work underscores the importance of rigorous chemical methodologies in identification of biologically active compounds. Holland *et al.* (1985) extracted 50 kg of barnacles, then processed the extracts through four sequential systems of thin layer chromatography. The active compound was detected by bioassay at each stage. The purified compound was derivatized for gas chromatography-mass spectroscopy (GC-MS), which yielded a single major GC peak. Mass spectra of derivatized hatching factor and hydrogenated derivatized hatching factor were consistent, not with a PG, but with another eicosanoid, 10,11,12-trihydroxy-5,8,11,17 eicosatetraenoic acid (Fig. 5). This compound is probably a lipoxygenase derivative of C20:5n3, an abundantly available fatty acid in marine invertebrates and also the precursor of the 3-series PGs.

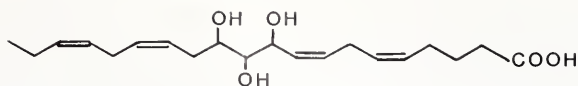


FIGURE 5. Structure of barnacle hatching factor, 10,11,12-trihydroxy-5,8,11,17-eicosatetraenoic acid.

Reproduction in Mollusca

PGs appear to stimulate egg production in the freshwater snail *Helisoma durgii* (Kunigelis and Saleuddin, 1986). When injected directly into the haemocoel of adults, ng quantities of PGE₂ produced apparent discomfort in all individuals and even death in isolated cases with no increase in egg masses or in eggs per mass. But when introduced into the female genital opening in a viscous fluid designed to approximate semem, PGE₂ treatments stimulated a long-term increase in egg production. Four weeks after treatment of virgin snails with 25, 50, and 100 ng doses of PGE₂, cumulative egg production was about 200, 425, and 650 eggs per animal, respectively.

Reproductive tissues from virgin and mated snails, the ovotestis, seminal vesicle, bursa copulatrix, and oothecal gland presented substantial PG-biosynthetic activity *in vitro*. Mating significantly altered the activity in two of the tissues. In ovotestis, synthesis of PGE₂ decreased while PGA₂ synthesis increased with no change in synthesis of PGF_{2α}. Synthetic activity changed in the bursa copulatrix, with PGE₂ and PGA₂ reduced to effective zero after mating; PGF_{2α} was again unchanged. Differences in PG-synthetic activity did not occur in seminal vesicle or oothecal gland (Kunigelis and Saleuddin, 1986). These two lines of evidence—the effects of PG treatments on egg production and alterations in PG-synthetic activity—suggest that PGs play important reproductive roles in this snail.

PGs are also produced by accessory sex glands of another snail, *Lymnaea stagnalis* (Clare *et al.*, 1986). Homogenates of the albumen gland, bursa copulatrix, prostate gland, and seminal vesicles converted radioactive arachidonic acid into labelled products that co-eluted with 6-keto-PGE₁, PGE₂, PGA₂/B₂ (not resolved), thromboxane B₂ (TxB₂), and several unknown compounds. Whole organs also converted arachidonic acid into these compounds, although in proportions different from the homogenates of the same organs. Effects of mating on PG-biosynthetic activity were not tested, nor were effects of PG administration on reproductive functions; nonetheless, the PGs formed in the reproductive organs eventually may be shown to play a still undefined role.

PGs induce spawning in two other molluscs, the abalone *Haliotis refescens* and the mussel *Mytilus californianus*. When added to seawater cultures at 3×10^{-12} M, PGE induced about a third and PGF about a half of male and female abalone to spawn (Morse *et al.*, 1977). Although the physiological mechanisms remain unclear, important biochemical insights have emerged. Addition of hydrogen peroxide to seawater tanks induced synchronous spawning in *H. refescens* and *M. californianus*. This observation is connected to the biochemistry of PG biosynthesis as understood in mammals. The first step in the conversion of arachidonic acid to the 2-series PGs is catalyzed by fatty acid cyclooxygenase (also known as prostaglandin endoperoxide synthetase). This involves first activation of the enzyme by a hydroperoxy group, then elimination of a hydrogen atom from C13 of arachidonic acid, leaving a free radical. This is followed by adding a peroxy radical in a bridge across C9 and C11, formation of the 8,12 carbon-carbon bond (required for the cyclopentane ring in the final prod-

uct), isomerization of the 11,12 double bond to 12,13, and addition of another peroxy radical to C15, with concomitant isomerization of the 12,13 double bond to 13,14. These final electron shifts generate PGG₂, a short-lived intermediate in the conversion of arachidonic acid to PG. The hydrogen peroxide effect is pH dependent, with lower concentrations releasing spawning at higher alkalinity. Morse *et al.* (1977) suggested that the alkaline conditions (pH 9.1) favored decomposition of hydrogen peroxide to the highly reactive hydroperoxy free radical. Since a hydroperoxy group activates the enzyme and peroxy radicals are added in two steps in the formation of PGG₂, the free radicals derived from hydrogen peroxide may enhance overall conversion of precursor fatty acids to PGs.

PGs appear to be important in basic physiological functions in molluscs, including ion regulation, possible renal function, and reproductive biology. This preliminary work sets the stage for important questions of the precise physiological activity, and offers the possibility of gaining greater understanding of invertebrate physiology and appreciation of PGs in these systems.

Oocyte maturation in starfish

Starfish oocytes develop to the first meiotic prophase, then await the spawning period. Maturation, or meiosis reinitiation, is induced by a hormone produced and released by the follicle cells surrounding the oocytes, 1-methyladenine. Once stimulated by the hormone, the oocytes complete the developmental path leading to fertilizable cells.

Arachidonic and eicosapentaenoic acids also induce oocyte maturation in three species of starfish: *Asterias rubens*, *Marthasterius glacialis*, and *Luidia ciliaris* (Meijer *et al.*, 1984). The PUFA-induced maturation is specific to these two fatty acids because 35 other fatty acids, ranging from C4:0 to C24:1 and including saturated, monounsaturated, and polyunsaturated fatty acids, did not induce maturation. The maturation effect is dependent upon extracellular calcium and occurs at physiological concentrations (*i.e.*, 50% maturation dose = 0.65 μ M arachidonic acid). The fatty acids stimulate the complete maturation program, including germinal vesicle breakdown, fertilization, and development into normal larvae. Fatty acids endogenous to the oocytes are able to stimulate maturation because addition of phospholipase A₂, an enzyme that hydrolyses the fatty acid from the beta-carbon of phospholipids, also stimulated maturation. The phospholipase effect was calcium-dependent, and specific because phospholipases C and D did not bring on maturation.

The hormone effect probably proceeds through release and metabolism of PUFAs. Two phospholipase A₂ inhibitors in mammals, quinacrine and bromophenacyl bromide, inhibit hormone-stimulated maturation, which can be overcome by increasing 1-methyl adenine concentrations. Five PGs did not stimulate maturation, and three cyclooxygenase inhibitors—acetylsalicylic acid, indomethacin, and tolazoline—did not inhibit maturation. On the other hand, three lipoxygenase inhibitors in mammals—quercetin, eicosatetraenoic acid and butylated hydroxytoluene—did inhibit hormone-induced maturation. Four products of lipoxygenase metabolism of arachidonic acid, 12- and 15-hydroxyeicosatetraenoic acids (HETE) and their corresponding hydroperoxyeicosatetraenoic acids (HPETE) stimulated maturation.

Oocytes convert radioactive arachidonic acid into HETEs (Meijer *et al.*, 1986a). Conversion of arachidonic acid does not occur in the absence of calcium, nor are oocytes stimulated to maturation. Following incubation with radioactive arachidonic acid, fractions with chromatographic behavior of HETEs were recovered and found to stimulate oocyte maturation. The lipoxygenase inhibitor eicosatetraenoic acid in-

hibited both conversion of arachidonic acid and stimulation of oocytes. It would appear, then, that 1-methyladenine acts by release of PUFA, followed by conversion to a biologically active HETE, which induces maturation of the oocytes.

Injection studies suggested that 12- and 15-HETE and corresponding HPETEs stimulated oocyte maturation (Meijer *et al.*, 1984). Upon re-evaluation, it was found that the tested compounds were contaminated with 5% of 8-HETE, the active compound in maturation (Meijer *et al.*, 1986a). Meijer *et al.* (1987) showed that (8R)-HETE, but not (8S)-HETE, is produced by starfish oocytes. The R isomer is the only active compound when tested in pure form, and other lipoxygenase products, including other HETEs and leukotrienes are not active.

A survey of eight starfish species shows that while the hormone 1-methyladenine stimulates maturation in all species, the stimulatory effect of arachidonic acid and 8-HETE occurs in only three of them (Meijer *et al.*, 1986b). Species differences in response to various eicosanoids also have been observed in various physiological settings in mammals. At this early period of appreciating the possible physiological activities of these compounds in invertebrates systems, species differences underscore the hazards inherent in forming generalizations.

Cercarial penetration of skin

Eggs of the blood fluke *Schistosoma mansoni* leave their mammalian hosts in urine or feces, and continue larval development in snails. Free-swimming larvae called cercariae reinfect mammalian hosts by burrowing through the skin or by ingestion with drinking water (Storer and Usinger, 1965). It has been known for a number of years that skin surface lipids stimulate cercarial penetration of animal membranes (Stirewalt, 1971). Among the skin surface lipids, free fatty acids, especially polyunsaturated fatty acids, appeared to be most efficacious in stimulating penetration (Austin *et al.*, 1972). Salafsky *et al.* (1984a) looked at the effect of certain fatty acids on two cercarial behaviors *in vitro*, namely cessation of swimming and initiation of penetration. Their results show that certain PUFAs attracted cercariae to the center of their test membranes while monounsaturated fatty acids did not. A few fatty acids gave intermediate results because two monounsaturated fatty acids were as stimulatory as the PUFAs, and two other monounsaturates were less stimulatory than the PUFAs but were clearly more stimulatory than controls. Cyclooxygenase metabolites, rather than the PUFAs *per se*, may alter cercarial behavior. Two inhibitors of cyclooxygenase—ibuprofen and, to a lesser degree, aspirin—inhibited cercarial response to PUFA. 13-Azaprostanoic acid, thought to specifically antagonize the platelet thromboxane/endoperoxide receptor in mammals, was also inhibitory.

PUFAs and certain of their metabolites may affect cercarial penetration as well as modify behaviors that precede penetration. When Salafsky *et al.* (1984b) compared cercarial penetration into skin membranes prepared from essential fatty acid (EFA) deficient and EFA replete adult rats, they found about three times less penetration in the preparations from EFA deficient rats. Again, the inhibition may be related to formation of eicosanoids. Interperitoneal injections of ibuprofen led to a time-dependent accumulation of the drug in the skin of EFA replete rats. Cercarial penetration of the drug-treated skin was reduced. The percent inhibition increased with increasing amount of ibuprofen accumulated in the skin, up to a maximum inhibition of about 84%.

When cercariae were incubated with radioactive linoleic acid, radioactivity could be recovered in high-pressure liquid chromatography fractions that eluted with PGE₂, PGD₂, LTC₄, LTB₄ and 5-HETE. These data suggest that cyclooxygenase and

lipoxygenase systems function within the cercariae. Radioimmunoassays of extracts from cercariae incubated with linoleic acid were also consistent with these products. Fusco *et al.* (1985) concluded that formation of eicosanoids is an essential step in penetration of human skin by cercariae of *Schistosoma mansoni*. If this can be supported by further work, it may present a rather interesting situation in which the PUFA substrate provided by a vertebrate host is metabolized into biologically active eicosanoids by a parasite.

It is not yet known how the eicosanoids alter the behavior of the cercariae or facilitate penetration of mammalian skin. Fusco *et al.* (1985) suggest that vasodilation, which is induced by certain PGs, may help the parasite find and infiltrate the blood system. It would appear that the eicosanoids, in this mode, would be usurped by the parasites to alter the host physiology. In this case, the finding by Rumjanek and Simpson (1980) that adult worms do not synthesize PGE or PGF may be appreciated in terms of host physiology. On the other hand, the behavioral effects of cessation of swimming and initiation of penetration (Salafsky *et al.*, 1984a), also induced by skin lipids, suggest a direct effect on the cercariae.

Sponge cell aggregation

Rich *et al.* (1984) suggest that the calcium dependent aggregation of marine sponge cells of *Microciona prolifera* is stimulated by leukotriene B₄ (LTB₄). LTB₄ induced rapid cell aggregation in a dose-dependent way at 0.2 and 1.2 μ M treatments. The effect appears to be specific for LTB₄ because eight PGs of A, B, D, E, and F series and eight lipoxygenase products failed to induce aggregation.

The calcium ionophore A23187 and the species-specific aggregation factor (MAF) stimulate cell aggregation. The aggregating effects of these compounds can be inhibited by cyclooxygenase inhibitors including nordihydroquaiaretic acid and indomethacin, which also interfere with calcium flux. These data show that those agents which inhibit calcium flux also inhibit aggregation while those that promote calcium movement also promote aggregation. Interpretation is difficult because while a specific lipoxygenase product promotes aggregation, inhibitors of cyclooxygenase metabolism inhibit it. Perhaps both pathways are involved in cell aggregation, with LTB₄ stimulating PG formation, which then acts in concert with the LTB₄.

Egg-laying behavior in crickets

The roles of PGs in insect reproduction were reviewed by Stanley-Samuelson and Loher (1986), from which the following summary is drawn. PGs were detected in extracts of various tissues from over a dozen species of insects. The most well understood physiological role of PGs is releasing egg-laying behavior in the field cricket *Teleogryllus commodus*. Adult females undergo sexual maturation, during which the abdomen becomes filled with hundreds of mature eggs. Certain behaviors that are likely to bring females into contact with males also develop. Insemination is achieved by transfer of a spermatophore to the genital organ of a female from where its contents migrate into the female's spermathecae. Cyclooxygenase activity is associated with the spermatophore contents, and once in the spermathecae of newly mated females, arachidonic acid is converted into PG.

It is not known how the PG formed in the spermatheca releases egg-laying behavior, but increases in spermathecal and hemolymph PG titer after mating suggest that the PG acts at some site distant from the source. The observations that PGE₂ does not stimulate contraction of oviduct muscles in *T. commodus* (Loher, 1984) nor in a

cockroach (Cook *et al.*, 1984) and that oviposition behavior is a complex activity directed by the central, rather than peripheral, nervous system (Loher, 1984) support the hypothesis that the PGs function at the level of the central nervous system.

Using egg-laying to assay structure-function relationships among a range of eicosanoids, Stanley-Samuelson *et al.* (1986) found that highest egg-laying activity was associated with E-series PGs. The A-, B-, D- and F-series induced zero to intermediate egg-laying. Structures that departed from the basic PG structure, represented by 15-HETE and prostacyclin, were inactive. The 2-series PGs were more active than their 1-series analogues; hence, there may be a biological specificity for PGE₂ in releasing egg-laying behavior in that particular cricket species.

Highest egg-laying activity was induced by 15-keto-PGE₂. In mammalian systems, this compound is formed by the action of prostaglandin dehydrogenase, located mainly in lungs, but also in liver and kidney. Biologically active PGE is rapidly cleared from the circulation of mammals by the activity of this enzyme. The observation that a biologically inactive compound, in the usual mammal assays, was associated with the greatest increase in egg-laying behavior marks a potentially important point in comparative physiology. Several features of the biology of eicosanoids appear to uniformly occur in the vertebrate and invertebrate systems as understood to date. For example, many compounds that inhibit the action of cyclooxygenase in mammals similarly inhibit the activity in invertebrates. On the other hand, as shown here, while the mammalian background will be important and useful in work on invertebrate systems, fundamental differences are to be expected.

PERSPECTIVES

Various eicosanoids appear to be involved in the regulation of a variety of physiological and behavioral areas in representatives of many invertebrate phyla. In some cases (such as mediation of behavioral thermoregulation), the evidence for an eicosanoid function is based on treatment of animals with a single compound and observation of the response. At this level of observation, it remains to be established that eicosanoids are physiologically involved. Given a good base of preliminary observations, important research goals would be to firmly show that, in the case at hand, PGs do mediate thermoregulatory behavior. In still other cases, such as the role of PG in releasing egg-laying behavior in crickets, there is sufficient evidence to accept that certain PGs do release egg-laying, although some details of the physiological mechanism—where in the central nervous system PGs act and how they alter behavior—are not yet understood. Research in this area could usefully be aimed, not at reaffirming the role of the eicosanoid, but at acquiring more details of the action. In study areas where considerable biochemical details are established—as in starfish oocyte maturation—cellular events remain unknown. Again, understanding how eicosanoids act remains a major research goal.

We are aware of eicosanoid roles in particular physiological areas in a given invertebrate organism. We know, for example, that PG releases egg-laying behavior in females of the cricket *T. commodus*. PGs are also detected in salivary glands, endocrine glands, Malpighian tubules, testes, and ventral nerve cords. Aside from the known role in altering behavior, what do PGs contribute to the other tissue systems in which they appear? Are they involved in regulating ion flux in Malpighian tubules, secretion in salivary glands, and neural function in the nerve cord, within the same organism?

Eicosanoids appear to be produced and to act at local, tissue, or cellular levels in mammals. PGE₁ produced by adipocytes functions within the same cells to modulate

the lipid mobilizing effect of certain hormones. Moreover, there are mechanisms that block PG circulation. The global circulation of PGE₂, for example, is checked by the action of prostaglandin dehydrogenase, located mainly in lungs, which converts the active compound into a biologically inactive product. However, in the cricket *T. commodus*, we speculated that the release of egg-laying behavior by PGE₂ is mediated in a mode more like a broadly circulating hormone (Stanley-Samuelson and Loher, 1986; Stanley-Samuelson *et al.*, 1986). This point can be extended to research in the mode of action, at the whole-organism level, of eicosanoids in invertebrates. The trihydroxyeicosatetraenoic acid that functions as hatching substance in the barnacle may be an example of a compound produced in one organ system with its action observed elsewhere, again suggesting hormone action.

With several likely roles of eicosanoids set forth, general research areas include establishing more firmly the activities, elucidating cellular details, and appreciating the possible modes of action of these compounds. One can assume that as details of eicosanoid action become known they will contribute greatly to our understanding of invertebrate physiology.

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INTERSPECIFIC AGGRESSIVE BEHAVIOR OF THE
CORALLIMORPHARIAN *CORYNACTIS CALIFORNICA*
(CNIDARIA: ANTHOZOA): EFFECTS ON SYMPATRIC
CORALS AND SEA ANEMONES

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ABSTRACT

Corallimorpharians are sessile cnidarians that are morphologically similar to the actinarian sea anemones and scleractinian corals. This study describes for the first time the behavioral mechanism and effects of aggression by a corallimorpharian. Polyps of the temperate clonal corallimorpharian *Corynactis californica* extruded their mesenteries and associated filaments onto members of certain species of sea anemones and corals. They did not exhibit this behavior intraspecifically, and members of different clones of *C. californica* remained expanded upon contact. In contrast, members of four species of corals and zoanthids responded to contact with *C. californica* by contracting their tentacles, and members of three sea anemone species bent or moved away, detached from the substrate, or attacked using their aggressive structures. When interspecific contact was prolonged, individuals of *C. californica* extruded filaments onto, and killed polyps of, the sea anemones *Anthopleura elegantissima* and *Metridium senile* within 3 weeks, and the corals *Astrangia lajollaensis* and *Balanophyllia elegans* within 4-10 months under laboratory conditions. The use of extruded mesenterial filaments by *C. californica* to attack members of other anthozoan species is similar to the aggressive behavior exhibited by many scleractinian reef corals. Field observations suggest that *C. californica* may use this agonistic behavior during interspecific competition for space on hard marine substrate.

INTRODUCTION

Some of the most striking behaviors exhibited by members of the class Anthozoa (Phylum Cnidaria) are the aggressive behaviors of certain actinarian sea anemones and scleractinian corals. Corals may attack competitors using sweeper tentacles (Richardson *et al.*, 1979; Wellington, 1980; Bak *et al.*, 1982; Chornesky, 1983; Hidaka and Yamazato, 1984), sweeper polyps (Sheppard, 1982), extruded mesenterial filaments (Lang, 1973; Glynn, 1974; Loya, 1976; Cope, 1981; Bak *et al.*, 1982; Logan, 1984), or nematocysts discharged from the colony surface (Rinkevich and Loya, 1983), and actinarian sea anemones may use elongated catch tentacles (Williams, 1975; Purcell, 1977) or marginal vesicles called acrorhagi (Bonnin, 1964; Francis, 1973b; Ottoway, 1978; Bigger, 1980; Brace, 1981; Ayre, 1982; Sebens, 1984). These aggressive responses are complex. They often involve the induced morphogenesis and directed application of specialized structures packed with nematocysts (Purcell, 1977; Chornesky, 1983; Watson and Mariscal, 1983; Hidaka and Yamazato, 1984; Hidaka, 1985), and may be initiated upon recognition of other genotypes or species of anthozoans (Lang, 1973; Bigger, 1980).

However, little is known about the aggressive behavior of another group of anthozoans, the corallimorpharians. Sebens (1976) reported the effects of competitive interactions between corallimorpharians and other anthozoans on the Caribbean coast of Panama, but did not specify the behaviors they used. The only other study relating to corallimorpharian behavior is that of Hamner and Dunn (1980), who described the unique feeding mechanism of some tropical Pacific corallimorpharians in which prey are enfolded in the oral disk.

Corallimorpharians occur throughout the world (Carlgren, 1949) and may be abundant on temperate rocky shores (Hand, 1955; Forster, 1958; Pequegnat, 1964; Castric-Fey *et al.*, 1978; Foster and Schiel, 1985), as well as on tropical coral reefs (Fishelson, 1970; den Hartog, 1980). Certain members of this group form clonal aggregations that cover large areas of hard substrate, and are the dominant sessile organisms in some temperate marine communities (Forster, 1958; Castric-Fey *et al.*, 1978). Thus, interactions of corallimorpharians with other sessile organisms may have important consequences for the structure of these communities.

Corallimorpharians superficially resemble the actinarian sea anemones in that they lack a calcareous skeleton (Carlgren, 1949). However, they are more like the stony corals in most other aspects of their morphology: they lack basilar muscles, may have tissue connections between adult polyps, lack ciliated tracts on their mesenterial filaments, and their cnidae are similar to those of corals (Carlgren, 1949; Schmidt, 1974; den Hartog, 1980). In light of the morphological relationships among members of these three anthozoan groups, a comparison of their aggressive behaviors is of interest.

This study describes the interspecific aggressive behavior of the temperate clonal corallimorpharian *Corynactis californica*. This behavior was first recorded in Chao's (1975) unpublished student paper. He observed that *C. californica* extruded mesenterial filaments to damage the sea anemones *Anthopleura elegantissima* and *Metridium senile* during interspecific interactions in the laboratory. This is aggression, which is defined by Webster's Third New International Dictionary as "an offensive action or attack," and in this instance is elicited upon contact with the anemones. Haderlie *et al.* (1980) briefly mentioned this behavior in their account of the natural history of *C. californica*. The present paper expands on these reports by presenting a quantitative analysis of mesenterial filament extrusion by *C. californica*, the specificity of this aggressive response, and its effect on the behavior and survival of some common sea anemones and corals under laboratory conditions.

Natural history

Corynactis californica Carlgren 1936 is the only species of corallimorpharian to occur along the west coast of North America, where it ranges from Washington State (Birkeland, 1971) to San Benitos Island in central Baja California (J. Engel, Tatman Foundation, pers. comm.). Members of this species reproduce asexually by fission (Hand, 1955) and budding (pers. obs.) to form aggregations on hard substrate (Fager, 1971; Haderlie *et al.*, 1980; Foster and Schiel, 1985), from the lower intertidal zone (Hand, 1955) to at least 50 meters depth (Birkeland, 1971; Schmieder, 1984, 1985). *C. californica* polyps are common on the vertical faces of subtidal rock reefs where they attain densities of up to 3000 polyps per square meter (Pequegnat, 1964). In southern California, field experiments show that the presence of aggregations of *C. californica* may increase the abundance of rock oysters (Vance, 1978) and mussels (Landenberger, 1967; Wolfson *et al.*, 1979) by protecting them from predation by sea stars. Groups of this corallimorpharian form interspecific boundaries with clones of

the sea anemones *Anthopleura elegantissima* and *Metridium senile* on artificial substrates such as wharf pilings (Chao, 1975; Haderlie and Donat, 1978) and offshore oil platforms (Carlisle *et al.*, 1964). Groups of *C. californica* also co-occur on subtidal rock reefs in kelp forests with several species of corals, most commonly the colonial coral *Astrangia lujallaensis* (Pequegnat, 1964) and the solitary corals *Balanophyllia elegans* and *Paracyathus stearnsii* (Pearse and Lowry, 1974; Lewbel *et al.*, 1981; Foster and Schell, 1985; North *et al.*, 1985).

Color varies greatly between different clonal aggregations of *C. californica*. Clones may be red, pink, orange, or occasionally blue or purple. Members of each aggregation asexually produce polyps of the same color in both the laboratory (pers. obs.) and the field (Turner *et al.*, 1969). Thus, color in this species appears to be genetically controlled, and in the present study polyps from different, distinctively colored aggregations were assumed to be genetically different (non-clonemates).

MATERIALS AND METHODS

Collection and maintenance of organisms

Specimens of *C. californica* and the other organisms used in this study were collected at four sites along the coast of central California (Table I). Laboratory experiments were conducted between June 1984 and July 1986 at three facilities of the University of California: Bodega Marine Laboratory, Joseph M. Long Marine Laboratory, and in cold rooms on the Berkeley campus. Organisms were maintained in plastic trays supplied with flowing seawater at ambient sea temperature (13–15°C), or in closed refrigerated aquaria filled with aerated natural seawater. All tanks were cleaned and animals fed adult brine shrimp (*Artemia salina*) weekly.

Mechanism and specificity of aggressive behavior

The first set of experiments focused on a description of mesenterial filament extrusion by *C. californica*, and determination of the stimuli that elicit this response. Only fully expanded, undamaged individuals of *C. californica* were used, and all within two weeks of collection. Polyps were brought into contact with a range of physical and biological stimuli (Table I) to elicit extrusion. Polyps were observed continuously for the first hour of contact, then once each hour for at least 12 h, and then intermittently for several days. A different individual of *C. californica* was used for each observation; Table I shows the number of replicate observations with each stimulus. Data were collected on the diameter and behavior of each polyp, occurrence of mesenterial filament extrusion, interval from the start of contact to extrusion, duration of extrusion, and the origin and maximal length of any extruded filaments.

Effects of C. californica on selected anthozoans

During the above contacts between polyps of *C. californica* and seven other species of anthozoans (Table I), data were also collected on the behavioral response of each anthozoan. Their responses to *C. californica* were categorized as: contracted, expanded, bent the column away, moved away on the pedal disk, detached from the substrate, or attacked *C. californica*. During trials between *C. californica* and mobile anthozoans such as the actiniarian sea anemones, the latter were repeatedly moved back into contact with *C. californica* to allow adequate time for a response.

The second set of experiments examined effects of *C. californica* on the survival of selected anthozoans over several weeks in the laboratory. To test the effect of *C.*

californica on actinarian sea anemones, individuals of the clonal anemones *Anthopleura elegantissima* and *Metridium senile* were placed in the center of groups of *C. californica* that were attached to shells or rocks. This method prevented movement away from contact by the anemones. Control anemones were placed on rocks that were interspersed in the same tray with experimental groups, but not in contact with *C. californica*. Data were then collected on the behavior and condition of the anemones once a week for three weeks.

Effects of prolonged contact with *C. californica* also were examined in two species of scleractinian corals, *Astrangia lajollaensis* and *Balanophyllia elegans*. Individual corals were attached to glass microscope slides or shells using H. A. Calahan's Marinepoxy (Davis Instruments, San Leandro, CA 94578). This epoxy has been used on anthozoans for several years in the laboratory without apparent harm (J. S. Pearse, University of California, Santa Cruz, pers. comm.). Barnacle shells bearing aggregations of *C. californica* were broken into small bits, and each piece of shell bearing a single polyp of *C. californica* was cemented adjacent to a coral. Empty shells with no *C. californica* were glued next to other corals as controls. Experimental and control plates of corals were then intermingled in trays of seawater, and the condition and behavior of each polyp was recorded once each month for 12 months. During this time, polyps of the asexually reproducing species budded off new individuals, and each month these were counted and the degree to which they had overgrown other polyps was determined.

RESULTS

Description of aggressive behavior in C. californica

Upon contact, the tentacles of individual *C. californica* adhered to those of polyps of certain other anthozoans. Then the interacting polyps often contracted slightly and their tentacles retracted. Over the next few minutes, the two polyps went through several cycles of expansion, contact, contraction, and re-expansion. If they maintained fairly constant tentacular contact, a mass of highly convoluted mesenteries and their associated filaments eventually appeared at the mouth or through a break in the body wall of the *C. californica* polyp (Fig. 1a). These filaments were withdrawn into the coelenteron at the end of each extrusion. Of 214 *C. californica* individuals observed, most extruded filaments through the mouth (69%), through openings in the column (7%), or along the junction of column and base (24%) of the polyp. One polyp put out filaments through the tips of its tentacles. These openings in the body wall were temporary and healed soon after the mesenterial filaments were withdrawn.

C. californica individuals almost always directed filaments laterally toward the side on which they had been stimulated (in 98% of cases, $n = 214$). These filaments then adhered to the source of stimulation and spread over its surface. They appeared highly extensible (Fig. 1b), and if the stimulus source was pulled away, the filaments could be stretched up to four times the diameter of the polyp to which they belonged. Extruded filaments ranged in length from 1 to 42 mm (median = 3 mm). However, most polyps extruded filaments only 1–10 mm in length (91% of polyps, $n = 190$), or about 0.1–1.5 times polyp diameter. Extrusion length did not vary with polyp size; small (5 mm diameter) polyps often extruded filaments at least 10 mm in length, while many large (>15 mm diameter) polyps put out filaments only 2–4 mm long. Often several mesenteries with their attached filaments were extruded by a single polyp, and they frequently spread to cover the organism that was the source of stimulation.



FIGURE 1. A. Side view of extrusion of mesenterial filaments by an individual of the corallimorpharian *Corynactis californica* onto a contracted polyp of the actinarian sea anemone *Metridium senile* (left). B. Top view of mesenterial filament extrusion by two polyps of *C. californica* (center) onto a retreating individual of the actinarian sea anemone *Anthopleura elegantissima* (upper right). Photo by Galen Rowell. Note that in both photographs the filaments extend toward the actinarians, and that in B they adhere to the anemone as it moves away. Scale bars = 1 cm.

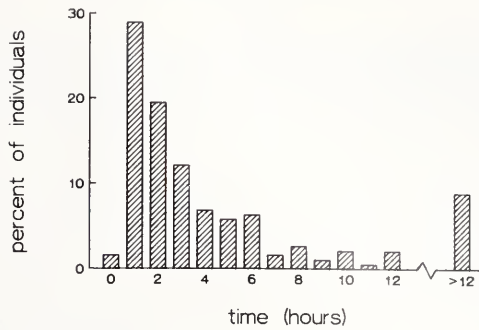


FIGURE 2. Time from start of contact to start of mesenterial filament extrusion by individuals of *Corynactis californica* upon contact with members of other anthozoan species and large food items ($n = 190$, median = 2.5 h, range = 0.5–72 h).

The interval from the start of contact to the start of extrusion ranged from 0.5 to 72 hours, but most individuals began to extrude filaments within a few hours of application of an appropriate stimulus (Fig. 2). At least 0.5 h of continuous contact was necessary to elicit extrusion; when contact was intermittent, extrusion often began only after several days. Most extruded filaments reached their maximal length 1–12 h from the start of contact (median = 7 h, range = 1–72 h, $n = 129$), and then were slowly withdrawn back into the coelenteron. The duration of extrusion varied greatly (median = 7 h, range = 1–144 h, $n = 149$); when contact with an appropriate stimulus was continuous, the filaments of some polyps remained extruded for up to six days.

Specificity of filament extrusion by C. californica

C. californica polyps extruded mesenterial filaments most frequently upon contact with certain types of biological stimuli (Table I). They did not respond to conspecifics, and instead, both clonemate and nonclonemate polyps remained expanded and intermingled their tentacles during contact. In contrast, a large percentage of *C. californica* individuals extruded mesenterial filaments onto members of three species of actinarian sea anemones and the scleractinian coral *Astrangia lajollaensis* (Table I). Extrusion onto the solitary corals *Paracyathus stearnsii* and *Balanophyllia elegans* was less frequent and often occurred only after 12 or more hours of contact. All ten polyps of *C. californica* that extruded filaments onto *P. stearnsii* did so 13–50 h from the start of contact, and extrusion was observed onto *B. elegans* only after several days or weeks from the start. Few *C. californica* individuals responded to the zoanthid *Epizoanthus scotinus* (Table I).

C. californica polyps rarely used mesenterial filaments to attack other sessile organisms such as hydroids, colonial tunicates, sponges, or algae (Table I). However, they did extrude filaments onto food items that were too large to ingest (Table I). To assess the size threshold for ingestion of large food items, expanded individuals of *C. californica* were offered pieces of fish that were less than, equal to, or slightly greater than their own polyp volume (by visual estimate). The polyps ingested food items that were smaller than or equal to their own volume in 38/49 cases (78%). When offered larger prey, however, they almost always extruded filaments over the food (Table I).

TABLE I

Collection sites, stimuli and percent of *Corynactis californica* that extruded mesenterial filaments onto each source of stimulus

Collection site	Stimulus	Common name	% <i>C. californica</i> that extruded filaments
ANTHOZOANS			
BCHM	<i>Corynactis californica</i> clonemates	Corallimorpharian	0 (28)
	non-clonemates		0 (40)
MB	<i>Anthopleura elegantissima</i>	Actinarian sea anemone	97.7* (44)
MB	<i>Metridium senile</i>	Actinarian sea anemone	89.4* (38)
B	<i>Epiactis prolifera</i>	Actinarian sea anemone	100* (17)
H	<i>Astrangia lajollaensis</i>	Scleractinian coral	88.9* (27)
CH	<i>Paracyathus stearnsii</i>	Scleractinian coral	43.5 (23)
H	<i>Balanophyllia elegans</i>	Scleractinian coral	6.5 (31)
C	<i>Epizoanthus scotinus</i>	Zoanthid	13.6 (22)
NON-ANTHOZOAN SESSILE ORGANISMS			
C	<i>Allopora californica</i>	Hydrocoral	23.5 (17)
C	<i>Garveia annulata</i>	Hydroid	0 (10)
H	<i>Acarinus erithicus</i>	Sponge	0 (28)
H	<i>Diaperoeia californica</i>	Bryozoan	11.1 (18)
H	<i>Archidistoma psammion</i>	Colonial tunicate	4.8 (21)
H	<i>Cystodytes lobata</i>	Colonial tunicate	13.3 (15)
H	<i>Rhodomenia pacifica</i>	Red alga	0 (21)
LARGE FOOD ITEMS			
MB	<i>Mytilus edulis</i>	Bay mussel	100* (21)
H	<i>Sebastes</i> spp.	Rock fish	83.0* (53)
PHYSICAL STIMULI			
—	Puncture with a glass needle**		4.8 (21)
—	Contact with a sterile glass rod		2.4 (42)

Numbers in parentheses indicate the number of polyps of *C. californica* exposed to each stimulus.

Collection sites: B = Breakwater at Doran Beach Park, Bodega Bay, Sonoma County, CA, on intertidal boulders, C = Cordell Bank, Marin County, CA, on rock pinnacles at 40–50 m depth, H = Hopkins Marine Life Refuge, Monterey County, CA, on rock reefs at 10 m depth, M = Monterey Municipal Wharf #2, Monterey County, CA, intertidally on wharf pilings.

* Responses significantly greater than those to all other stimuli, G-test for homogeneity of replicates, $G = 14.08$, $P < .05$.

** The column of each polyp of *C. californica* was punctured with a sterile glass needle, which was left in place for at least 12 h.

Differences were observed in the quality of extrusion onto food items versus anthozoans. When presented with large pieces of fish or mussel, most *C. californica* expanded, pressed their oral disks and tentacles onto the food, and extruded filaments out through their mouths (Table II). In contrast, when contacting anthozoans such as sea anemones or corals, *C. californica* often contracted and/or put out filaments laterally through openings in the body wall (Table II). Filaments extruded onto anthozoans also were significantly longer than those extruded onto prey items (Fig. 3).

In response to physical contact with an inert glass rod, or physical damage to the column wall, *C. californica* rarely extruded filaments (Table I). However, polyps did put out filaments when subjected to extreme physical stress, such as when they were accidentally crushed or became desiccated. Individuals also occasionally extruded filaments in the absence of any apparent stimuli.

TABLE II

Comparison of behavioral responses to different stimuli (food items versus anthozoans) by *Corynactis californica* during extrusion of mesenterial filaments

Stimulus	Posture: Filament origin:	Number of <i>C. californica</i> with each type of response during extrusion			
		Expanded		Contracted	
		Mouth	Body wall	Mouth	Body wall
Large food items (fish, mussel)		63	1	1	0
Anthozoans (corals, zoanthids, sea anemones)		24	29	54	23

See Table I for species of stimuli used.

The distribution of the responses is dependent upon the type of stimulus contacted ($R \times C$ test of independence using G-test, $G = 62.67$, $P < .01$).

Effects of *C. californica* on the behavior and survival of other anthozoans

Contact with polyps of *C. californica* caused strong avoidance or attack responses by most of the anthozoans tested (Table III). However, conspecific *C. californica* of different genotypes (non-clonemates) did not avoid each other, and most remained expanded during contact. Non-mobile anthozoans of other species, such as scleractinian corals and zoanthids, contracted their tentacles and often their entire polyps within minutes when placed in contact with *C. californica* (Table III). Two individuals of the coral *Paracyathus stearnsii* extruded their mesenterial filaments at 7 h but these did not extend far enough to contact or damage *C. californica* polyps. The actiniarian sea anemones varied in response depending upon whether they were surrounded by *C. californica* polyps. When not surrounded, most individuals of *Anthopleura elegantissima* and *Metridium senile* bent away, moved away via pedal locomotion, or attacked the corallimorpharian (Table III). Three polyps of *A. elegantissima* inflated their specialized aggressive structures called acrorhagi and applied them to *C. californica* at 0.5–2 h. These attacks left acrorhagial peels that caused localized

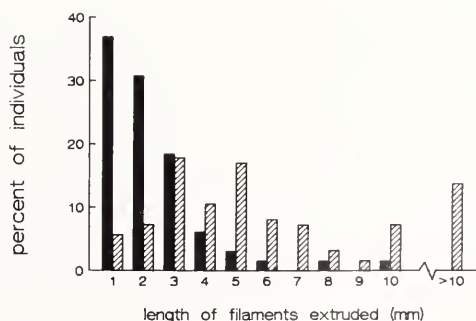


FIGURE 3. Comparison of length of mesenterial filaments extruded by *Corynactis californica* in response to large food items (shaded bars, $n = 65$, median = 2 mm, range = 1–10 mm) versus anthozoans (striped bars, $n = 123$, median = 5 mm, range = 1–42 mm). A significant difference exists between the two populations (normal approximation to the Wilcoxon rank sum test, $Z = 6.83$, $P < .01$). See Table I for species used.

TABLE III

Variation in the behavioral responses of selected anthozoans to contact with polyps of the corallimorpharian *Corynactis californica*

Type of anthozoan	Total number tested	Number with each behavioral response					
		Expand	Contract	Bend away	Move away	Detach base	Attack
NON-CLONEMATE CONSPECIFICS							
OF <i>C. californica</i>	40	38	2	0	0	0	0
CORALS	106	12	92	0	0	0	2 ^a
ZOANTHIDS	20	0	20	0	0	0	0
SEA ANEMONES							
<i>Anthopleura elegantissima</i>							
not surrounded	34	0	0	2	29	0	3 ^b
surrounded	15	4	6	0	0	5	0
<i>Metridium senile</i>							
not surrounded	22	0	1	2	8	1	5 ^c + 5 ^d
surrounded	18	3	1	0	0	5	9 ^c
<i>Epiactis prolifera</i>							
not surrounded	14	0	0	1	5	8	0

Types of attack: a = extrusion of mesenterial filaments by the coral *Paracyathus stearnsii* at 7 h; b = acrorhagi; c = extruded acontia; d = catch tentacles, used by 5/10 individuals that possessed them. See Table I for species of corals and zoanthids used.

Not surrounded/surrounded indicates whether or not each anemone was surrounded by polyps of *C. californica* during the interaction.

The distribution of responses was dependent upon the type of anthozoan involved ($R \times C$ test of independence using G-test, $P < .01$).

damage to *C. californica* polyps, but the damaged areas healed within a few days. Five out of ten polyps of *Metridium senile* that possessed well-developed aggressive structures (catch tentacles) also inflated and applied them to polyps of *C. californica* within 2–11 h of contact. However, none of these catch tentacles adhered to the corallimorpharians, and they did not appear to cause damage. *Metridium senile* also frequently extruded acontia onto *C. californica*, both when surrounded and not surrounded (Table III). The acontia adhered strongly to and killed some *C. californica* individuals. Polyps of the actinarian sea anemone *Epiactis prolifera* were tested only when not surrounded, and most avoided contact within 3 h by moving away on the substrate or detaching their pedal disks and then rolling or floating away (Table III).

Individuals of the sea anemones *M. senile* and *A. elegantissima* were killed within one to three weeks (Fig. 4a) during prolonged contacts with surrounding groups of *C. californica* polyps. These anemones often detached from the substrate, but adhered to the tentacles of the surrounding *C. californica* polyps and were unable to escape. They were then repeatedly attacked by the extruded mesenterial filaments of *C. californica*, and their tissues became necrotic within a few days. Control anemones that did not contact *C. californica* remained expanded and firmly attached to the substrate throughout the experiment.

Corynactis californica had a much slower but fatal effect on members of two species of scleractinian corals. Within two weeks from initial contact, *C. californica* had caused tissue damage to most individuals of the corals *Astrangia lajollensis* (57/74 polyps damaged, =77%), and *Balanophyllia elegans* (12/19 polyps damaged, =63%).

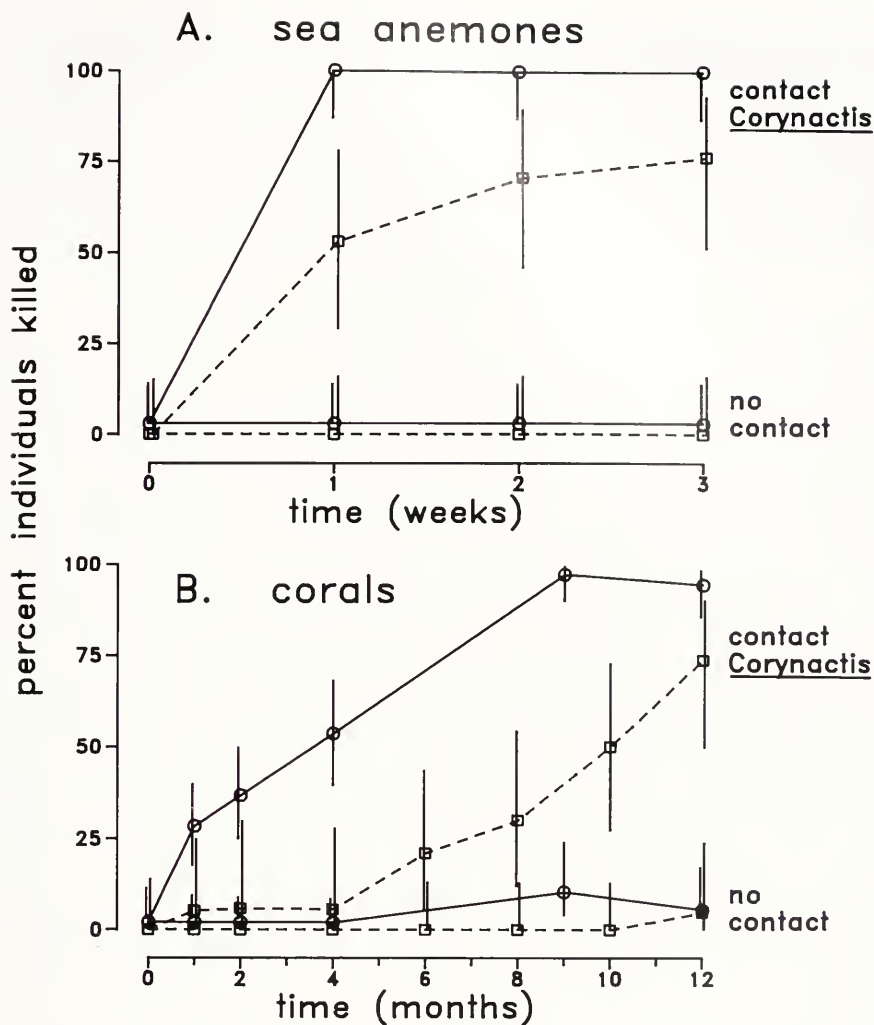


FIGURE 4. Effect of contact with the corallimorpharian *Corynactis californica* on the survival of selected sea anemones and corals. Bars represent 95% confidence limits. See text for details. A. Effect on polyps of the actiniarian sea anemones *Anthopleura elegantissima* (solid lines, $n = 21$ contact, $n = 20$ no-contact) and *Metridium senile* (dashed lines, $n = 17$ contact, $n = 17$ no-contact). At three weeks, the proportions of anemones killed in the experimental (contact) versus control (no-contact) groups were significantly different for both species (G-test of independence for proportions, $P < .01$). B. Effect on polyps of the scleractinian corals *Astrangia lajollaensis* (solid lines, $n = 69$ contact, $n = 31$ no-contact) and *Balanophyllia elegans* (dashed lines, $n = 19$ contact, $n = 21$ no-contact). At twelve months the proportions of corals killed in the experimental (contact) versus control (no-contact) groups were significantly different for both species (G-test of independence for proportions, $P < .01$).

During the ensuing months, *C. californica* polyps asexually produced many new individuals which eventually grew over and around the corals. In 6 months on one plate, 10 *C. californica* individuals produced over 80 polyps that killed and covered the original 10 *A. lajollaensis* polyps. After 12 months of contact, corallimorpharian polyps had killed most of the corals on the experimental plates (Fig. 4b).

C. californica individuals appeared to affect only the corals that they touched. In several cases, tissue was damaged and calcareous skeleton was exposed only on the side of a coral that faced toward a *C. californica* polyp. In cases where asexually produced polyps of *C. californica* grew away from and ceased to contact the experimental corals, the latter remained healthy and undamaged. Control corals that were isolated from contact with *C. californica* also remained alive (Fig. 4b), and during the year produced many new polyps, presumably both sexually (via brooded planulae) in *Balanophyllia elegans*, and asexually (via clonal budding) in *Astrangia lajollaensis*.

DISCUSSION

This report is the first detailed description of aggressive behavior in a corallimorpharian. The type of aggression exhibited by *C. californica*, extrusion of mesenterial filaments, is very similar to the attack behavior of many tropical scleractinian corals (Lang, 1973; Glynn, 1974; Loya, 1976; Wellington, 1980; Cope, 1981; Bak *et al.*, 1982; Logan, 1984). *C. californica* and certain corals readily extrude their mesenterial filaments onto members of other anthozoan species and onto large food items (Table I; Yonge, 1930a; Lang, 1973). The timing of the extrusion response is also remarkably similar in corals and *C. californica*. Lang (1973) reported that Jamaican reef corals extruded their filaments 0.5–12 h after initiation of contact with certain coral species, and Glynn (1974) noticed extrusion by eastern Pacific corals 8–12 h after contact with competing corals. In the present study, most *C. californica* individuals also put out their filaments within 12 h (Fig. 2). The extruded filaments of both *C. californica* and scleractinian corals cause extensive damage to and eventually kill other anthozoans if contact is prolonged and if, in corals, the other colony is small enough (Lang, 1973; Fig 4). Since corals and corallimorpharians are morphologically very similar (den Hartog, 1980), one might expect to see this similarity in their aggressive behaviors as well. This type of aggression, via mesenterial filament extrusion, differs from the competitive behavior of some of the actiniarian sea anemones that coexist with *C. californica* and use their marginal spherules (Francis, 1973b) or catch tentacles (Purcell, 1977) to attack competitors. These differences in behavior underscore the major morphological differences between a corallimorpharian such as *C. californica*, and actiniarian sea anemones. They also support the idea that corallimorpharians are more closely related to scleractinian corals than they are to sea anemones.

Unlike the specialized aggressive structures of actiniarian sea anemones that are used only during competitive interactions (Bonnin, 1964; Francis, 1973b; Williams, 1975; Purcell, 1977; Watson and Mariscal, 1983), the mesenterial filaments of *C. californica* appear to serve a variety of functions. In all anthozoans studied thus far, the mesenterial filaments are the major sites for digestion and absorption of food in the coelenteron (Yonge, 1930b; Nicol, 1959; Van-Praet, 1985). These filaments contain gland cells that secrete strong proteolytic enzymes, as well as nematocysts that may inject cytolytic toxins into prey (Van-Praet, 1985). Special areas on the filaments and adjacent mesenteries then absorb the partially digested foodstuffs (Yonge, 1930b; Van-Praet, 1985). *Corynactis californica* also extrudes mesenterial filaments onto food that is too large to take into the coelenteron (Table I), presumably to digest it externally. This behavior allows polyps to consume a large range of prey sizes. Certain tropical Pacific corallimorpharians envelope prey in the oral disk, and then extrude filaments out of the mouth to digest them (Hamner and Dunn, 1980). Many species of reef-building corals also consume prey externally via extruded filaments (Carpenter, 1910; Yonge, 1930a, 1968; Goreau *et al.*, 1971). Thus, two major functions of

mesenterial filaments in these organisms appear to be the internal breakdown and absorption of food, and external consumption of large prey. Mesenterial filaments also are used during physical stress. Some corals extrude their filaments when oil is introduced into their coelenterons (Bak and Elgershuizen, 1976), when they are exposed to intense light (Lang, 1973), or when they are handled roughly (Duerden, 1902). *C. californica* polyps also exhibit extrusion when stressed (see Results). Finally, divers observed *C. californica* polyps extruding their mesenterial filaments onto one of their major predators, the sea star *Dermasterias imbricata* (Annett and Pierotti, 1984; pers. obs.). Thus, the lobed filaments along the edges of anthozoan mesenteries may serve multiple functions in certain corals and corallimorpharians.

An interesting aspect of the aggressive/defensive use of mesenterial filaments by *C. californica* is the complete lack of response to conspecifics (Table I). Members of a given clonal aggregation presumably would benefit from damaging and overgrowing those of a different, genetically distinct aggregation (as discussed by Francis, 1973b). However, in the field distinctly colored groups of *C. californica* often intermingle and show no evidence of aggression or damage along their interacting borders (pers. obs.). The wide, anemone-free zones that are visible between aggregations in other species known to show interclonal aggression (Francis, 1973a; Purcell, 1977) do not occur in this species. Most reef corals that use mesenterial filaments to attack competitors also only extrude them interspecifically (Lang, 1973; Cope, 1981). One exception has been reported: the Caribbean coral *Montastrea annularis* appears to extrude filaments onto conspecific colonies to damage them (Logan 1984, 1986).

The present study demonstrates that under laboratory conditions *C. californica* strongly affects both the behavior and survival of certain other anthozoans (Table III, Fig. 4). These results have several ecological implications. In shallow subtidal habitats along the coast of California where *C. californica* occurs, hard surfaces are often completely covered with organisms (Pequegnat, 1964; Haderlie and Donat, 1978; Vance, 1978; Schmieder, 1984, 1985), and space for settlement and growth may be a limiting resource for sessile animals. The species of sea anemones tested in this study often moved away from or otherwise avoided contact with *C. californica* (Table III); where they co-occur in the field, this behavior might free space for growth along the interspecific borders of *C. californica* aggregations. The avoidance responses of these sea anemones are the same behaviors used to effectively escape attack by conspecifics (Francis, 1973b; Purcell, 1977) and predators (Waters, 1973; Edmunds *et al.*, 1976). However, the specialized aggressive structures of *Anthopleura elegantissima* and *Metridium senile* apparently were not effective against *C. californica* (see Results). The acontia of *M. senile* caused the most damage to *C. californica*, and in the field may allow the former to kill polyps of the latter along their interacting borders. L. Harris (University of New Hampshire, pers. comm.) has observed that, in the laboratory, *M. senile* also uses acontia to attack individuals of the sea anemones *A. elegantissima*, *Actinia equina*, and *Urticina* (= *Tealia*) *piscivora*.

The results of the present study differ somewhat from those presented by Chao (1975). He described an aggressive hierarchy in which *C. californica* was dominant over *A. elegantissima*, while *M. senile* was dominant over both of the former species. The present results confirm that *C. californica* causes tissue damage to *A. elegantissima* (Table III, Fig. 4a), but show that *C. californica* and *M. senile* damage each other, with no clear competitive outcome. A clear dominance ranking of these three cnidarian species remains to be determined.

Field observations also suggest that *C. californica* damages sea anemones and corals under natural conditions. Chao (1975) noticed that interspecific boundary areas about 2–3 cm wide occurred between aggregations of *C. californica* and the sea anem-

ones *Anthopleura elegantissima* and *Metridium senile* found on intertidal pilings at the Monterey Wharf. Francis (1973b) and Purcell (1977) showed that anemone-free zones between groups within the latter two species are maintained by aggression between clones. The corridors along their boundaries with *C. californica* could be maintained by the avoidance behaviors of the anemones (Table III), or by the death of anemones that have been repeatedly attacked by the mesenterial filaments of *C. californica* (Fig. 4a).

On subtidal rock reefs, certain scleractinian corals also appear to be negatively affected by contact with *C. californica*. Fadlallah (1981) observed a polyp of *C. californica* extruding filaments onto and killing an individual of the solitary coral *Balanophyllia elegans* in the kelp forest at Hopkins Marine Life Refuge (HMLR) in Monterey. Polyps of *B. elegans* and the colonial coral *Astrangia lajollaensis* that occur adjacent to *C. californica* on subtidal boulders at HMLR often show damaged tissues and exposed skeletons (pers. obs.). In addition, the vertical distribution of *C. californica* and *A. lajollaensis* on large subtidal reefs suggests some sort of negative interaction. Pequegnat (1964) found that *C. californica* was most abundant near the top of a subtidal reef in southern California, and became more sparse with depth. In contrast, *A. lajollaensis* formed large colonies near the base of the reef and decreased in abundance with height, occurring at low densities near the reef top. These inverse patterns of abundance also can be observed on large (2–5 m high) subtidal reefs at HMLR in central California (pers. obs.). Efforts are currently underway to document the distributions of these anthozoans at HMLR, and to test the ecological effects of their behavioral interactions in the field.

Members of the genus *Corynactis* produce clonal aggregations in tropical and temperate marine habitats throughout the world (Carlgren, 1949). *Corynactis viridis* is the most abundant sessile organism on shallow subtidal rocks walls at the Glenan Archipelago on the Atlantic coast of France (Castric-Fey *et al.*, 1978), and at Plymouth, England (Forster, 1958); *C. parvula* occurs on Caribbean reefs where it may interact with a variety of corals and sea anemones (den Hartog, 1980). These congeners may show aggressive behavior similar to that of *C. californica*, as well as use other competitive mechanisms (den Hartog, 1977), thus affecting the abundance and distribution of co-occurring sessile organisms.

Many so-called lower animals exhibit complex aggressive behaviors associated with resource defense. Such behaviors are observed in polychaete worms (Evans, 1973; Dimock, 1974; Roe, 1975), chitons (Chelazzi *et al.*, 1983), limpets (Stimson, 1970; Branch, 1975; Wright, 1982), sea urchins (Schroeter, 1978; Maier and Roe, 1983), and sea stars (Menge and Menge, 1974; Wobber, 1975), as well as in the many anthozoans discussed in this paper. Yet the behaviors of these organisms are rarely considered in theoretical works on aggression and territoriality, most of which focus on birds and mammals (Waser and Wiley, 1979; Murray, 1981; Davies and Houston, 1984), nor are they included in recent texts on animal behavior (Huntingford, 1984; Ridley, 1986). Because marine invertebrates often are sessile or slow-moving, and may be clonal as well, their aggressive behaviors have developed under a different set of constraints than have those of most vertebrates. More extensive consideration of aggression in the lower invertebrates may lead to important new insights into the evolution and ecology of animal conflict.

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DEVELOPMENT, METAMORPHOSIS, AND SEASONAL ABUNDANCE OF EMBRYOS AND LARVAE OF THE ANTARCTIC SEA URCHIN *STERECHINUS NEUMAYERI*

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ABSTRACT

The development to metamorphosis of the shallow-water antarctic sea urchin, *Sterechinus neumayeri*, is described for the first time. Developmental stages are similar to those of closely related temperate species with feeding larvae, but the rate of development is extremely slow. Hatching of ciliated blastulae occurs approximately 140, 128, and 110 hours after fertilization at -1.8 , -1.0 , and -0.5°C , respectively, more than twice the time required for closely related temperate species near their normal ambient temperature. Larvae reared at -1.8 to -0.9°C are capable of feeding 20 days after fertilization and are competent to metamorphose after 115 days. Early cleavage embryos, blastulae, gastrulae, and prism larvae of this species were collected from the plankton adjacent to McMurdo Station, Antarctica, in early November and December, 1984 and 1985. Echinoplutei were not found during this study, but they have been collected from the plankton in other years; there is no evidence that the larvae are demersal. The timing of spawning ensures that feeding larvae are in the plankton during the abbreviated summer peak of phytoplankton abundance in McMurdo Sound. Recruitment of juveniles into the benthos most likely occurs in synchrony with the subsequent period of high levels of benthic chl *a* concentrations.

INTRODUCTION

The perception that brooding is the prevalent mode of development among species of antarctic echinoderms has been firmly established over the past century (Thomson, 1876; Thorson, 1950; Mileikovsky, 1971; Dell, 1972; White, 1984). Brooding is most apparent within the shallow-water echinoid faunas (Arnaud, 1974; Picken, 1980). Three families in three separate orders (Cidaridae, order Cidaroidae; Schizasteridae, order Spatangoida; and Echinidae, order Echinoidea) represent the antarctic echinoids. Two of the three families are dominated by species that brood. Fell (1976) reported that 12 of 19 known species of antarctic cidarids are known brooders, and 3 others almost certainly brood. In addition, females of all 21 known species of antarctic schizasterids brood their young in specialized sunken aboral petaloids known as marsupia (Fell, 1976).

It is unclear whether the high incidence of brooding species among these two families is a consequence of ongoing selection in the antarctic environment or of phylogenetic history (Dell 1972; Fell, 1976; Arnaud, 1977). Fell (1976) hypothesized that the ancestral forms of antarctic cidarids (ancestral goniocidarids) brooded their young, and he suggested that cidarids colonized the antarctic as brooders. No ancestral form has been clearly established for antarctic schizasterids. Since extant non-

antarctic representatives of this group have unprotected development, the brooding habit of antarctic species may have evolved subsequent to their colonization of the antarctic (Fell, 1976). In either case, the numerical success (*i.e.*, number of species) of cidarids and schizasterids in the antarctic apparently is related—at least in part—to their brooding habits.

In contrast to the cidarids and schizasterids, the antarctic echinids are represented by only five species, all within a single genus, *Sterechinus* (Fell, 1976). Individuals of one species, *S. neumayeri*, are the most abundant echinoids in shallow-water surrounding the antarctic continent. The relatively small maximum egg sizes reported for three antarctic species of *Sterechinus* (0.15 in *S. neumayeri*, and 0.25 mm *S. agassizii* and *S. antarcticus*) are indicative of a free-swimming mode of development (Mortensen, 1909, 1910; Pearse and Giese, 1966). Moreover, despite frequent collections, brooding has not been reported for any of the six species of the genus (Fell, 1976). The absence of post-spawning parental care among antarctic representatives of this group is in sharp contrast with the predominant mode of development in other antarctic echinoids.

Little is known about the embryonic and larval stages of non-brooding antarctic echinoids. Mortensen (1913) described echinoplutei from plankton samples collected by the German South Polar Expedition (1901–1903). Mortensen assigned the larvae to *S. neumayeri* because it was a common species, had very small eggs, and was not known to brood. A pair of echinoplutei was collected from midwater in McMurdo Sound by the British National Antarctic Expedition (MacBride and Simpson, 1908). Mortensen (1913) also assigned these to *S. neumayeri*. Since the publication of these reports over 70 years ago, little additional information on the developmental stages of non-brooding antarctic echinoids has been obtained. Pearse and Giese (1966) described the reproductive cycle of a population of *S. neumayeri* in McMurdo Sound, and suggested that the larvae of this species are demersal and not pelagic because they have been taken from the plankton so rarely; however, the larval development of antarctic echinoids had not been observed or described.

The present paper describes the development through metamorphosis of *Sterechinus neumayeri*, and draws special attention to the slow rates of embryonic and larval development. In addition, we present information on the seasonal abundance of embryos and larvae of this species in the near-shore waters of McMurdo Sound, Antarctica.

MATERIALS AND METHODS

Individuals of *Sterechinus neumayeri* were collected by scuba divers from 15–25 m depth beneath the annual sea ice adjacent to McMurdo Station, Antarctica (77° 51' S, 166° 40' E). In November 1983, immediately after collection, approximately two dozen animals were transported to the University of California, Santa Cruz, where gametes were fertilized and the larvae were reared through metamorphosis in an ice bath (−0.5 to 0.5°C) kept in a 4°C refrigerated unit. Additional studies of the developmental stages and developmental rate of *S. neumayeri* were carried out at McMurdo Station; ripe animals were collected in November, 1984, and larvae were reared through metamorphosis and early juvenile stages to December, 1985. The running seawater system at McMurdo Station maintains aquarium seawater temperatures between −1.8 (winter) and −0.9 (summer)°C, which allowed us to rear embryos and larvae close to their ambient temperatures.

Spawning and fertilization of gametes

Spawning was induced by intracoelomic injection of 0.5 M KCl solution. Eggs collected from 10–15 spawning females were washed in clean 5 μ m filtered seawater and mixed with a few drops of dilute sperm suspension in a 4 liter polycarbonate culture vessel. After approximately 20 minutes, eggs were filtered off with 20 μ m nitex screening and placed in 4 liter culture vessels with clean 5 μ m filtered seawater.

Rearing of embryos and larvae

Embryos and larvae were reared in gently stirred and unstirred cultures (Hinegardner, 1969; Strathmann, 1971). The water in the culture vessels was changed every four days using a 20 μ m mesh nitex strainer to retain the embryos and larvae.

At McMurdo Station, larvae were fed semi-daily with equal amounts of bacterized cultures of *Isochrysis galbana* and *Phaeodactylum tricornutum* (total concentration 10,000–15,000 cells/ml), which were grown at 15°C in continuous light using half strength F medium (Guillard and Ryther, 1962). Algal samples to be used as food were centrifuged for 10 minutes at 5000 rpm and resuspended in clean filtered seawater (–1.5°C). Phytoplankton concentrations were measured using a Palmer Maloney counting chamber.

Initially, at Santa Cruz, several phytoplankton species (including both temperate and antarctic forms) were tested as potential sources of food for the larvae. Among five temperate species tested (*Amphidinium carteri*, *Dunaliella tertiolecta*, *Isochrysis galbana*, *Phaeodactylum tricornutum* and *Rhodomonas* sp.), *I. galbana* and *P. tricornutum* were most resistant to low temperatures. These phytoplankton appeared to be healthy, even after being in larval cultures for two days, and were readily consumed by the larvae. Cells of the antarctic diatom *Thalassiosira antarctica* were not readily ingested by early stage plutei.

Settlement and metamorphosis of larvae reared in Santa Cruz was induced by adding echinoplutei to glass dishes containing pieces of PVC pipe covered with a bacterial-algal film (Hinegardner and Tuzzi, 1971). The bacterial-algal film was prepared by placing the PVC pipe in a large dish that was held in a running seawater table for several days. Competent echinoplutei reared at McMurdo Station were successfully induced to settle and metamorphose with sediment samples collected from various depths (12, 20, 25, and 30 m) within the adult habitat.

Embryonic developmental rates

Time of development to hatching at different temperatures was determined for embryos reared at McMurdo Station by holding them in culture vials that were (1) in a refrigerated unit at –0.5 (–0.7 to –0.3)°C, (2) in a running seawater table in the laboratory at –1.0 (–1.2 to –0.9)°C, and (3) submerged in the sea 5 m below the level of the sea ice at ambient temperature, –1.8 (–1.9 to –1.7)°C (a small heated hut with a hole in the floor and through the sea ice was used as a staging area). Approximately 50 newly spawned eggs from a pair of females and a single drop of dilute sperm suspension were mixed in each of 3 sets of 10, 5 ml capacity vials filled with 5 μ m filtered seawater at the appropriate temperature. Progress of development and incubation temperature were monitored every 12–16 hours during early cleavage stages and every 2 hours near the time of hatching. Because agitation and small changes in temperature may adversely affect rates of embryonic development, only previously undisturbed culture vials were used for observations of developing embryos. The time

of hatching was defined as the time when at least 10% of the ciliated blastulae in a particular incubation vial were released from the fertilization membrane.

Field collection of embryos and larvae

Plankton samples were collected on a weekly or bimonthly basis from September, 1984 to December, 1985 using both diver-towed and stationary current-fed plankton nets (240 μ m mesh) at various locations in McMurdo Sound. The conical, stationary nets measured 2 m in length with a circular mouth opening of 0.3 m. The diver-towed net was 2 m long and had a rectangular mouth of 0.1×0.3 m. Each of the current-fed nets was held open continually by a steel frame; net buoyancy was regulated with a float. Two or three nets were attached to a weighted steel cable and suspended by scuba divers from the undersurface of the sea ice for 24 to 48 hours. At the points of attachment to the cable, the nets had a ball bearing swivel which allowed them to orient to the shifting directions of the prevailing currents.

Because the larvae may be demersal, 5 replicate bottom cores of 8 cm diameter were taken monthly from October, 1984 through October, 1985 at 10, 20, 25, and 30 m depth adjacent to McMurdo Station.

All samples were sorted for larvae and other organisms within two days of collection. Early developmental stages that were not readily identifiable were isolated from field samples and reared in the laboratory until they reached a recognizable larval stage. Sizes of embryonic and larval stages as well as larval skeletal morphology of the field-collected specimens were noted and compared to those of embryos and larvae reared in the laboratory from fertilization.

RESULTS

Sequence of development

Development of *Sterechinus neumayeri* was followed through metamorphosis at Santa Cruz (-0.5 to 0.5°C) and McMurdo Station (-1.8 to -0.9°C) (Table I). The eggs are small (mean diameter = 0.179 mm; $n = 55$) and negatively buoyant. Early development yields a typical sea urchin prism larva. Stomadeal breakthrough occurs 20 days after fertilization at approximately -1.5°C , and soon thereafter the larvae begin to feed. By the 21st day, the postoral and anterolateral paired arms of the echinopluteus are formed. The larval epithelium is now sparsely covered with red pigment granules, more or less randomly distributed. Formation of the posterodorsal and the much shorter preoral pair of arms begins at approximately 43 and 56 days after fertilization, respectively. The onset of the eight-arm pluteus stage is closely timed with the formation of the anterior epaulettes as well as the appearance of the five lobes of the hydrocoel (Fig. 1). At this stage of development the larvae are similar to those previously described from collections of earlier antarctic expeditions (MacBride and Simpson, 1908; Mortensen, 1913).

Further thickening of the ciliary band along the posterior margin of the larva results in the formation of the posterior epaulettes. By approximately the 80th day of development at -1.8 to -0.9°C , the tube feet primordia are formed. Soon thereafter, a variable number (1–3) of triradiate spines appear on the external surface of approximately 40% of the larvae. The most conspicuous of the spines is located in a medial position at the posterior end of the larva, while the other two are formed on the right side, near the bases of the postoral and posterodorsal rods of the larval skeleton.

Metamorphosis is relatively slow, lasting 2–3 hours before the non-feeding benthic juvenile is formed. Newly metamorphosed juveniles retain many of the pigment

TABLE 1

Developmental stages and approximate sizes of developmental stages of *Sterechinus neumayeri* reared in Santa Cruz (ice water at -0.5 to 0.5°C) and McMurdo Station, Antarctica (-1.8 to -0.9°C)

Developmental stage	Size (mm)	First appearance (days)	
		-1.8 to -0.9°C	-0.5 to 0.5°C
Fertilized egg	.18-.19	—	—
Blastula	.21	2.1	1.7
Hatching	—	5.1	3.7
Gastrula	.22	10	8
Prism	.32	16	15
Early pluteus	.35	21	17
Six-arm pluteus	.54	43	29
Early eight-arm pluteus	.80	56	42
Late eight-arm pluteus	1.20	103	100
Juvenile	.44	115	107

Sizes represent the diameter of ova, blastulae and juveniles, maximum length of gastrulae and prism larvae, and length from the aboral apex to the tips of postoral arms of echinoplutei.

granules characteristic of larval stages, but otherwise have a pale, whitish appearance. They have a single set of well developed tube feet as well as 10 juvenile and 15 primary spines. The triradiate spines which appeared on the surface of echinoplutei are retained on the aboral surface of juveniles.

Duration of embryonic development

Embryos reared below the sea ice (-1.9 to -1.7°C), in a seawater table (-1.2 to -0.9°C), and in a refrigerator (-0.7 to -0.3°C) at McMurdo hatched at 140, 122, and 110 hours, respectively. Time to first hatching for embryos reared in an ice bath at Santa Cruz (-0.5 to 0.5°C) was approximately 88 hours.

Occurrence of eggs, embryos and larvae in the plankton

One hundred and twenty (120) plankton samples were taken from McMurdo Sound between September, 1984 and December, 1985. Of these, 56 were taken from near the undersurface of the ice or, in the absence of sea ice, near the surface of the water. Fourteen were taken from midwater (10–20 m depth), and 50 were collected from near the bottom at 15–30 m depth.

Large numbers (500–600) of embryos, free-swimming blastulae, and gastrulae that closely resembled those of laboratory reared *Sterechinus neumayeri* were collected from the plankton at all depths sampled using both stationary and diver-held plankton nets. Eggs and early stage embryos were collected predominantly during the third and fourth weeks of November, 1984 and 1985. Hatched blastulae and gastrulae at various stages of development were predominant during the first week of December, although several unhatched and newly hatched blastulae were collected from surface waters on the 9th of November, 1985. Four prism larvae were identified from midwater samples taken in mid to late December, but no echinoplutei were collected during this study. No sea urchin eggs, embryos, or larvae were found in the 240 bottom cores collected and examined.



FIGURE 1. Early eight-arm pluteus of *Sterechinus neumayeri* shortly after the formation of the pre-oral pair of arms (indicated by arrow). Scale bar = 100 μ m.

DISCUSSION

Embryonic and larval development

Compared to other species that have been studied, the developmental stages of *Sterechinus neumayeri* are most similar in shape and size to those of the temperate echinoid, *Echinus esculentus* (MacBride, 1903). However, the formation of spines on the external surface of the larvae, separate from the juvenile rudiment, clearly distinguishes the larvae of *S. neumayeri* from those of *E. esculentus* and other species studied within the family Echinidae (MacBride, 1903; Arrau, 1958; Cram, 1971). Morphologically similar spines reportedly develop on the echinoplutei of several other species of regular echinoids, including both euechinoid and cidaroid forms (Onoda, 1931, 1936; Fukushi, 1960; R. Emlet, pers. comm.).

The time of development for the entire period from fertilization to metamorphosis of *Sterechinus neumayeri* is extremely long. Within the family Echinidae, the tem-

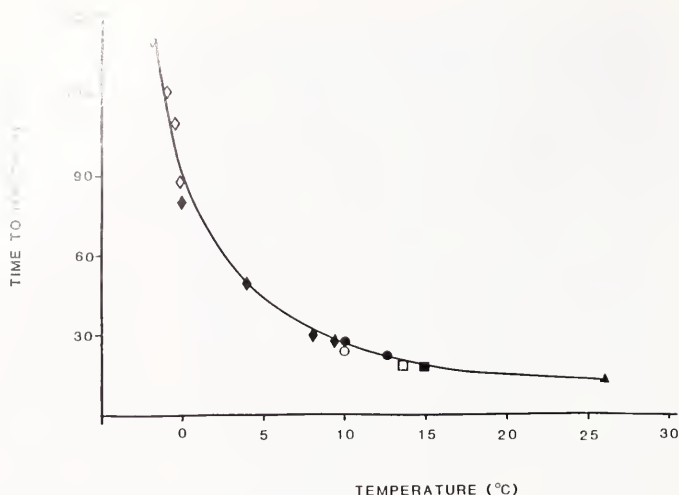


FIGURE 2. Duration of embryonic development to hatching as a function of temperature for seven species of echinids and stronglylocentrotids with indirect development. Hatching occurs at the ciliated blastula stage. Mean diameter of ova ranges between 80 (*Strongylocentrotus purpuratus*) to 179 μm (*Sterechinus neumayeri*). ♦ *Strongylocentrotus droebachiensis* reared at 0, 4, 8°C (Stephens, 1980) and 9–10°C (Strathmann, 1974); ● *S. franciscanus* reared at 10, and 12–13°C (Strathmann, 1974); ▲ *S. pulcherrimus* reared at 25–27°C (Onoda, 1936); ○ *S. purpuratus* reared at 10°C (Strathmann, 1974); □ *Loxechinus albus* reared at 13–14°C (Arrau, 1955); ■ *Parechinus angulosus* reared at 15°C (Cram, 1971); ◇ *Sterechinus neumayeri* reared at -1.9 to -1.7, -1.2 to -0.9, -0.7 to -0.3, and -0.5 to 0.5°C (this study).

perate species *Parechinus angulosus* and *Psammechinus miliaris* are competent to metamorphose 60 days after fertilization at ambient temperatures (10–16°C) (Shearer *et al.*, 1913; Cram, 1971), less than half the time required for *S. neumayeri* near their normal ambient temperature (-1.5°C). This observation agrees with the general trend noted by Emlet *et al.* (in press) between decreased temperatures (and increased latitudes) and increased time to metamorphosis for echinoids with planktotrophic larvae.

Because factors unrelated to temperature may influence rates of post-embryonic development (*e.g.*, larval food and density, Kume and Dan, 1968; Hinegardner, 1969), we critically compared the rates of embryonic development to the hatched blastula stage at different temperatures, both of *Sterechinus neumayeri* and other sea urchin species with planktotrophic larvae within the families Echinidae and Strongylocentrotidae. Time to hatching ranged from a minimum of 13 hours at 25–27°C in the tropical species *Strongylocentrotus pulcherrimus* to a maximum of 140 hours at -1.9 to -1.7°C for *S. neumayeri*, and was intermediate for temperate species near their normal ambient temperatures. The duration of embryonic development to hatching for these seven echinoid species is a curvilinear function of temperature, with increased sensitivity at lower temperatures (Fig. 2). A direct relationship between the duration of embryonic development and temperature has been found with interspecific comparisons among other poikilotherm groups, including asteroids (Pearse, 1969), amphipods (Bregazzi, 1972), barnacles (Patel and Crisp, 1960), copepods (McClaren *et al.*, 1969), and rotifers (Herzig, 1983b). Moreover, studies on single species or physiological races reveal the same function, describing the immediate thermodynamic effect of temperature on developmental processes [See for example,

Bougis (1971), Stephens (1972), and McEdward (1985) for temperate echinoids; Herzig (1983a) for copepods; Herzig (1983b) for rotifers; and Ross and Quetin (1986) for antarctic krill]. The direct relationship between temperature and duration of embryonic development, both within a single species and among groups of related species, suggests that there is little or no temperature compensation for developmental rates in poikilotherms, resulting in the observed general trend of increasingly longer periods of development with greater latitude.

The tendency for increased lecithotrophic development among high latitude marine invertebrates was well documented by Thorson (1950) who proposed that the combination of low temperatures—which act to increase development time—and a short season of phytoplankton abundance in high latitude environments select against planktotrophic larvae. Thorson's (1950) explanation has been challenged by several authors. In particular, Underwood (1974) and Clarke (1982, 1983) argue that there should be no *a priori* reason to expect ontological processes to be rate-limited by temperature because all poikilotherms have evolved the capability to modify those processes for the effects of temperature. However, although numerous mechanisms for metabolic temperature compensation have been identified (Hochachka and Somero, 1984), there are few examples of developmental rate compensation for temperature in any previous work (Clarke, 1982). Development is a complex, highly synchronized process involving many biochemical and structural changes. As suggested by Patel and Crisp (1960), basic patterns of temperature-developmental rate interactions may not be readily modified in evolution.

Seasonal abundance and distribution

The presence of embryonic and early larval stages of *Sterechinus neumayeri* in the plankton during early to mid November and December, 1984 and 1985 is in accordance with previous estimates of the spawning time of this species in McMurdo Sound (Pearse and Giese, 1966). Observations of spawning urchins further substantiate this conclusion: males spawned in shallow water near McMurdo Station on two occasions during the first week of November, 1984 (B. Gullikson and T. Klinger, pers. comm.). Coupled with known development times of laboratory-reared embryos and larvae, this evidence suggests that larvae of *S. neumayeri* feed between late December and early March, coinciding with the summer peak of phytoplankton abundance in McMurdo Sound (Bunt, 1964; Rivkin *et al.*, 1986). Consequently, settlement of larvae onto the benthos will occur predominantly during late February and March, in synchrony with the annual period of high benthic chl *a* concentration that occurs during the austral Fall (Berkman *et al.* 1986).

Twenty-five plankton tows and 16 bottom cores were collected and examined between late December and early March, 1984–1985, yet no echinoplutei of *Sterechinus neumayeri* were found. Littlepage (1966, 1968, and pers. comm.) collected and analyzed 547 plankton samples taken throughout the year from McMurdo Sound but found no echinoderm larvae. The conspicuous absence of echinoplutei from plankton samples taken over areas where adult *S. neumayeri* are abundant led Pearse and Giese (1966) to suggest that the embryos and larvae of this sea urchin are demersal. However, large numbers of *S. neumayeri* embryos and early larvae were collected from the water column during this study. Moreover, echinoplutei of this species have been taken from the antarctic plankton in other years: all 48 specimens recorded by MacBride and Simpson (1908) and Mortensen (1913) were taken from the water column; in addition, four echinoplutei of *S. neumayeri* were collected from near-surface waters, over approximately 300 m of water, in early January, 1986 (Rivkin *et*

al., 1986). This demonstrates that embryos and larvae of *S. neumayeri* are readily carried off the bottom by currents. Given the active swimming behavior of echinopluteus in laboratory cultures (I. Bosch, pers. obs.), it is unlikely that development of this species is demersal. More extensive, multi-annual sampling is needed to provide conclusive evidence on the larval distribution of *S. neumayeri*.

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FEEDING ADAPTATIONS OF THE FORAMINIFERAN *CIBICIDES REFULGENS* LIVING EPIZOICALLY AND PARASITICALLY ON THE ANTARCTIC SCALLOP *ADAMUSSIUM COLBECKI*

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ABSTRACT

The calcareous foraminifer *Cibicides refulgens* is a conspicuous and abundant component of the epifaunal community living on the valves of the free-swimming Antarctic scallop, *Adamussium colbecki*. Examination of this association using light microscopy, scanning electron microscopy, radiotracer, and resin-casting/sectioning techniques, demonstrates that the foraminifer possesses a combination of morphological and physiological adaptations, unique among benthic calcareous foraminifera, which enhance its ability to acquire nutrients in an otherwise oligotrophic and seasonal environment. Three distinct modes of nutrition are employed: (1) grazing the algae and bacteria living upon the scallop shell surface, (2) suspension feeding through the use of a pseudopodial net deployed from a unique superstructure of agglutinated tubes which form an extension to the calcareous test, and (3) parasitism by eroding through the scallop's shell, and using free amino acids from the highly concentrated pool in the extrapallial cavity.

INTRODUCTION

A variety of benthic foraminiferal species are known to live most, or part of their lives, epizoically on a wide range of organisms. Examples include *Rosalina globularis* d'Orbigny, *R. anomala* Terquem, *R. carnivora* Todd, *Cibicides refulgens* Montfort, *C. lobatulus* Walker and Jacob, *C. pseudoungerians* (Cushman), *Discorbis wrighti*, and *Discorbinnella* sp. which firmly attach to macroscopic algae and metazoans such as hydroids, bryozoans, tunicates, crustaceans, isopods, amphipods, decapods, pycnogonids, brachiopods, gastropods, and bivalves (Nyholm, 1961; Todd, 1965; DeLaca and Lipps, 1972; Hayward and Haynes, 1976; Zumwalt and DeLaca, 1980; Mullineaux and DeLaca, 1984; Alexander, 1985; Moore, 1985).

The most important association in terms of numbers of foraminifera appears to involve filter feeding invertebrates, particularly free-swimming bivalves. Hayward and Haynes (1976) reported 998 individual foraminifers on one specimen of the commercial scallop *Clamys opercularis* (Linnaeus) of which 765 were *Cibicides lobatulus*. Similarly, Mullineaux and DeLaca (1984) noted an average of 1386 foraminifers on 21 specimens of the Antarctic scallop *Adamussium colbecki*, 901 of which were *C. refulgens*.

For the majority of associations between filter feeding invertebrates and epizoic

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foraminifers, the host shell may provide not only a firm substrate for attachment, but also the added advantage of a relatively silt-free environment. In motile bivalves living in areas of strong currents and wave action, the shell may provide further protection against sand shifting and possible burial with fatal consequences (Dobson and Haynes, 1973; Hayward and Haynes, 1976). Even sessile molluscs such as *Mytilus* can provide a relatively silt-free substrate (Allen, 1953), and *Notocorbula* living in the silt-laden Mississippi delta, offers a preferred habitat for *Hanzawaina* sp. by crawling above the layers of accumulating flocculent material (Bock and Moore, 1969). Furthermore, life activities of the host can enhance the availability of nutrients to the epizoic foraminifers; for example seven species living on the shell of the brachiopod *Tichosina floridensis* Cooper, are thought to benefit from suspended food material transported by the inhalant and exhalant feeding currents (Zumwalt and DeLaca, 1980).

The means by which foraminifera attach themselves to the shells of their hosts varies considerably. Some adhere simply, with no detectable effect or marking on the substrate (Bock and Moore, 1969; Zumwalt and DeLaca, 1980), while others extensively pit and erode the subsurface layers (DeLaca and Lipps, 1972) and even penetrate the entire shell to reach the mantle cavity (Todd, 1965).

In this paper we use radiotracer techniques, light and scanning electron microscopy, resin casting, and sectioning methods to describe the remarkable morphological and physiological adaptations of a large Orbitoidacean, *Cibicides refulgens*, to its specialized epizoic habit on the free-swimming Antarctic scallop, *Adamussium colbecki*.

The study site

The study site at Explorers Cove (approximately 77.6 S, 163.5 E), McMurdo Sound, Antarctica, was used previously by Stockton (1984) and Mullineaux and DeLaca (1984).

The sediment is homogeneous fine silt mainly deposited from the late austral summer/early autumn freshwater input to the locality via streams originating from the Commonwealth and Wales glaciers (DeLaca, unpub. obs.). The virtual absence of currents (Mullineaux and DeLaca, 1984) prevents any reworking of the sediments and results in a seasonal accumulation of fine silt. *Adamussium colbecki* occurs in densities of up to 90 m⁻² and forms 90% of the available hard substrate in the area (Stockton, 1984); it resides within depressions in the sediment which are caused by the light, non-locomotory flapping action of its valves (Mullineaux and DeLaca, 1984; Stockton, 1984).

The benthic community of Explorers Cove is thought to resemble the deep sea in species diversity, abundance of individual organisms, sediment relief, and long-term stability of temperature, salinity, and oxygen (Dayton and Oliver, 1977). The austral summer is accompanied by an increase in primary productivity by ice algae living within the lower 10 cm of sea ice, and is followed in late summer by release of the algae into the water column from the melting ice. Thus a seasonal pulse of organic material is contributed to the developing *in situ* productivity in shallow-water (DeLaca, unpub. data).

MATERIALS AND METHODS

Living specimens of *A. colbecki* were collected from 20 to 27 m by scuba diving through holes blasted in the 3 m thick sea ice, and maintained in an aquarium at *in situ* temperature and salinity (−1.8°C and 34‰ S).

Most material used for scanning electron microscopy (SEM) was coated with gold and/or carbon and examined in a Cambridge Stereoscan MK 2 operated at 20 kV; images were recorded on Ilford FP4 35mm film developed in Microphen.

Scallop shells with epifaunal communities were embedded in Spurr's low viscosity resin (Polysciences, Inc.), then ground and polished using carborundum, diamond, and aluminum oxide abrasives until the desired plane of section was reached. Azure II/methylene blue, and methylene blue in borax (Richardson *et al.*, 1960) was used to stain organic material. Other scallop shells, cleared of epifauna by boiling in 20 vol. % hydrogen peroxide, were gradually embedded in Spurr's over three days to penetrate the fine cavities of the shell material. After polymerization, the block was fractured along the plain of the shell so that the upper block retained only a thin translucent layer of calcite. Alternating treatments of 0.1 *N* HCl at room temperature, and 3% aq. sodium hypochlorite at 60°C, removed calcite and organic layers, respectively; the exposed face of the lower block forms a perfect cast of the scallop shell dorsal surface and the canals and cavities within the calcite itself. Observations were made with an ETEC Autoscan SEM at the Wadsworth Center for Laboratories and Research, Albany, New York.

For SEM examination of substrate pitting, agglutinated tube morphology, and pseudopodial deployment, six scallops with epifauna were fixed for 2 h in 6% glutaraldehyde buffered with 0.1 *M* sodium cacodylate at pH 7.4. Dorsal valves were rinsed five times with distilled water, rapidly frozen at -40°C, and freeze dried in an inverted position. Other *C. refulgens* were picked from all size classes of *A. colbecki* (Stockton, 1984) and examined for gross morphology, aperture, and spiral face detail; attachment zones were fractured and the exposed calcite laminae studied for evidence of pitting or tunneling. Etched (using 0.1 *N* HCl) and non-etched inner valve surfaces were examined for perforations.

To determine the rate of substrate pitting and agglutinated tube formation, foraminifera were picked from the dorsal valves of *A. colbecki*, cleaned of extrathalamic material, and placed in semi-enclosed, transparent plastic chambers attached to areas of non-pitted dorsal valves from recently killed scallops (50% of which had most of their microflora removed). It was not possible to use living *A. colbecki* since the frantic flapping of collected specimens prevented the introduction and subsequent attachment of *C. refulgens* to the upper valves. Such violent movements are not usual in the normal habit of *A. colbecki* (Mullineaux and DeLaca, 1984; Stockton, 1984). The valves with experimental foraminifera were returned to the collection site for 3 months; the containing chambers did not alter ambient light levels and a loose fitting lid prevented silt accumulation but permitted exchange of dissolved materials.

ATP analysis (DeLaca, 1986) was used to distinguish living from dead foraminifera and to measure foraminiferal biomass. A carbon to ATP ratio of 300 was tentatively assumed for application to ATP values from *C. refulgens*, since recent work shows that this ratio is remarkably constant between the two taxonomically distant rhizopod species, *Gromia oviformis* (order Testacida) and *Astrammina rara* (order Foraminiferida) (DeLaca, 1986). Cellular nucleotides were extracted with phosphate/citrate buffer at 100°C, and data were used for normalizing experimental results.

Labeled amino acids (^{14}C) in the same proportions as a typical algal protein hydrolysate (Amersham corporation, product CFB.25; see table I), were used to demonstrate uptake through the pecten shell by individual foraminifera. Plastic containers (12 ml volume) were sealed to the inner valve surface with silicon vacuum grease, and the seawater within enriched with 2.5 μCi radio-labeled amino acids at 100 μM final concentration. This concentration of amino acids is approximately 25 times lower than that recorded for free amino acids within the extrapallial cavity (see Re-

sults) and 14.7 times greater than at the sediment/water interface (DeLaca, 1982). Incubations lasted up to four days, and controls with containers on both faces of the shell (one contained label; the other retained any diffused label) measured leaching through non-pitted shell and passive diffusion into heat killed (30°C for 30 min), attached foraminifers. To establish the viability of animals harvested, 10 specimens from each experimental group were analyzed for ATP content.

To measure influx rates of dissolved amino acids, individual animals were removed from pecten shells, cleared of all extraneous materials, and allowed to recover from handling for 24 hours prior to experimentation. These animals were incubated in experimental medium [10 ml filter sterilized seawater (FSSW) with labeled and unlabeled compounds (depending upon experiment)]. Incubated specimens were washed in 5–6 serial baths of FSSW (~1 min each) until wash water registered no significant radioactivity over background levels. Influx was determined by measuring the level of accumulated radioactivity in experimental animals (homogenized in Aquasol 2) with a Beckman LS 6800 liquid scintillation counter. “Time zero” and heat killed controls were used.

To measure grazing rates of *C. refulgens*, epiflora of the dorsal valves were labeled *in situ* with [^{14}C] sodium bicarbonate in light at temperatures between -1.8 and 0°C for 12 hours; individual cleaned and heat killed *C. refulgens* were placed on these shells prior to labelling. After incubation, the scallop shells with foraminifers were washed in serial baths of FSSW until the radioactivity of wash water was not significantly over background levels. Twenty individual diatoms, living foraminifers, and heat killed foraminifers were selected as time-zero samples and extracted in 1.0 ml of hot (100°C) phosphate/citrate buffer. After removal of $10\ \mu\text{l}$ of supernatant for ATP analyses, the extract was dried and digested with $0.3\ \text{ml}$ of $0.2\ \text{N}$ perchloric acid prior to adding Aquasol 2. Subsequent specimens were sampled at three 6-h intervals, and similarly processed. Radiation counts obtained from the time zero specimens were subtracted, and the results used to calculate the number of cells ingested. Experimental protocols for isolating, washing, and determining ^{14}C uptake by single algal cells were taken after Rivkin and Seliger (1981).

Suspension feeding was investigated using radiolabeled bacteria and diatoms. Bacteria were isolated from the sediments of New Harbor and further isolated on 2216 Marine Agar (Difco). Selected cultures were then labeled with [^{14}C] leucine (ICN) at log growth in Marine Broth (2216 Difco). Labeled bacteria were washed free of extraneous label by repeated centrifugation and resuspension in FSSW, until supernatant radioactivity was not significantly over background levels. Cell concentrations were determined with a Petroff-Hauser counting cell, and disintegrations per cell measured by liquid scintillation using Aquasol 2. *Nitzschia cylindricus* cultures, provided by Dr. C. W. Sullivan (University of Southern California), were grown with Alga-grow media (Carolina Biol. Suppl. Co.) in FSSW and labeled with [^{14}C] sodium bicarbonate. These cultures were concentrated and washed on nitex screen and resuspended to the desired concentration (measured using a plankton counting cell).

Four dorsal valves of living *Adamussium colbecki* were removed, and the agglutinated portions of 50 *C. refulgens* were gently but thoroughly removed from the test, leaving the foraminifers securely attached to the shells. Two of the shells were maintained at temperatures between -1.8 and 0°C , and the others were warmed to 30°C for 30 min (heat killed controls); shells were suspended upside down for 6 h in a culture vessel with a suspension (maintained with a small stream of air) of labeled diatoms in seawater. This configuration was duplicated to measure bacterial capture. At t_0 and hourly intervals, samples of both diatom and bacterial suspensions were taken (by centrifugation and filtration with nitex screen, respectively) to check for

dissolved label and to measure cell concentration. Following incubation, 30 foraminifers with agglutinated tubes, and 30 foraminifers without, were detached, cleaned of extraneous material (if necessary), washed through 10 serial washings of FSSW, extracted in ortho-/phosphate buffer, and processed as described above.

Cell dimensions were measured using light microscopy. Approximate cell volumes were calculated by appropriate geometric formulae corresponding to the cell shape. Carbon content was also calculated (Strathmann, 1967).

Small volumes of fluid were sampled from the extrapallial cavity of *A. colbecki* (between the mantle and inner surface of the shell) by passing a blunt cannula attached to a syringe through a window cut in the 0.5–0.7 mm thick shell. Separation, identification, and quantification of free amino acids in selected samples were accomplished using high pressure liquid chromatography (HPLC) after fluorescence derivatization with ortho-phthaldialdehyde (see Stephens, 1982).

RESULTS

The dorsal surfaces of *Adamussium colbecki* living in sedimentary environments are encrusted with attached foraminifera (Fig. 1) including *C. refulgens*. The force required to dislodge an individual of *C. refulgens* from the surface of a bivalve shell increases with size of the individual, as does the extent and depth of substrate erosion. Juvenile *C. refulgens* (Fig. 2) are easily dislodged using a fine needle, whereas adult specimens (Figs. 3–6) must be pried off with a stout microprobe. A random sample of shell surfaces under *Cibicides refulgens* demonstrated that only 45% ($n = 200$ on each of 5 shells) of foraminifers had caused etching. Similar sampling near the umbo revealed that 92% of the foraminifers resided in etched concavities whereas only 12% of those foraminifers nearer the shell margins were attached over etched shell (100 foraminifers examined on each of 5 shells). No significant etching was detected after three months on shells artificially infested with *C. refulgens* (Fig. 7).

A progression in the extent of substrate pitting caused by increasing sizes of foraminifers and the age of the shell is clearly visible using a dissecting microscope. Use of the SEM demonstrated that pits caused by younger *C. refulgens* generally extend no deeper than the uppermost laminae of the valve. At this stage the striations visible on the shell surface (Stockton, 1984) and the smaller perpendicular 'ribs' connecting them (Fig. 8) are removed completely in the area of the pit, and the exposed calcite is eroded to appear as irregular granules with multitudinous 'micro-canals' (Figs. 9, 10). Further pitting results in enlargement of the microcanals to form distinct canal openings which penetrate several calcite layers, and are to some extent guided by planes of weakness within, or between, the layers (Figs. 11, 13). This phenomenon is dramatically illustrated by resin casts which show the pit erosion in reverse, producing a 'cathedral effect' from the pattern of channels within the scallop shell material (Fig. 12). The distributions of canal openings within the substrate pits as a whole do not exhibit any noticeable pattern.

The bond between the test wall of the umbilical face of *C. refulgens* and the uppermost calcite layer in the pit is sufficiently strong so that when a specimen is forcefully detached from the shell, a layer of shell material will often remain attached to the foraminifer. It is then possible to observe deeper canals ramifying through the shell; these canals are generally fewer in number than the more superficial canals, but are larger in diameter (10–14 μm) and more conspicuous.

Fracturing a scallop shell directly through a substrate pit allows for detailed SEM study of groups of canals in the middle and lower layers (Fig. 22). Scanning electron micrographs (Figs. 13, 14, 16) demonstrate conclusively that the canals do not funda-

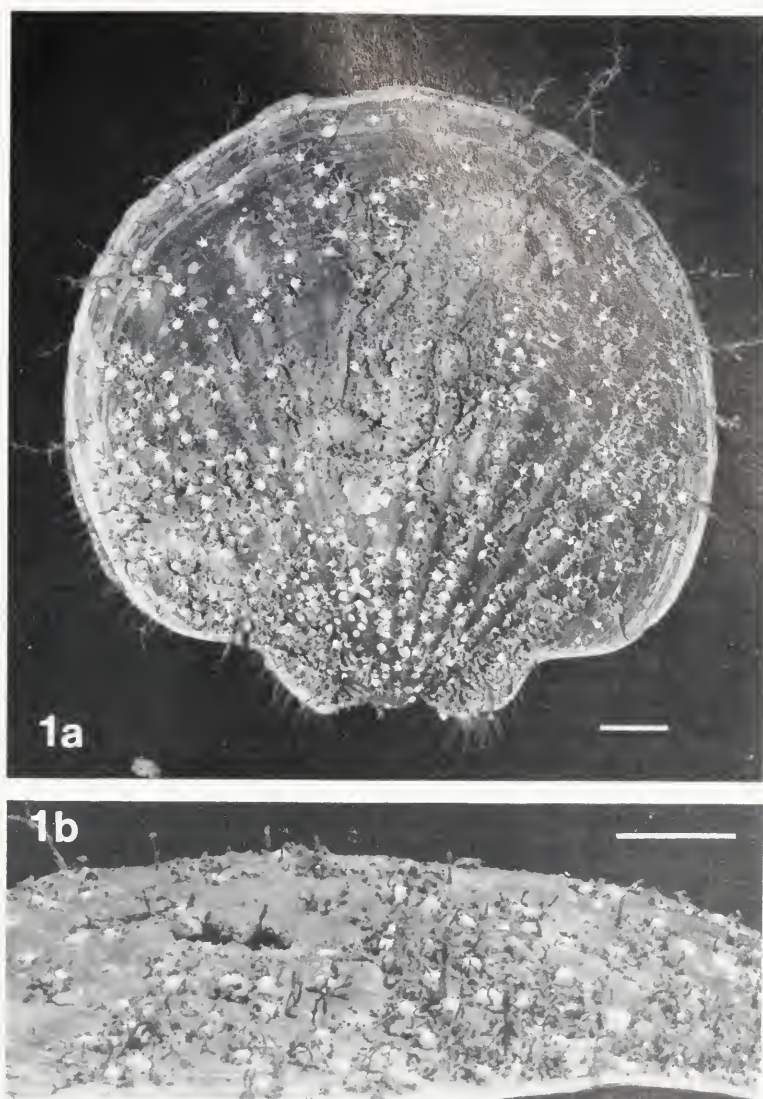


FIGURE 1a. The free swimming Antarctic scallop *Adamussium colbecki* with characteristic epizoic growth. Conspicuous attached faunal components include the agglutinated tube of a large polychaete, hydrozoans, bryozoans, and commonly four or more species of benthic foraminifera. The most abundant and conspicuous species is *Cibicides refulgens* with its agglutinated tubes. Scale bar = 1 cm. b. Oblique view of the dorsal valve of *A. colbecki* with attached *C. refulgens* and associated agglutinated tubes reaching into the overlying water. Vertical tubes may extend to 5 mm and exhibit three orders of branching. Scale bar = 5 mm.

mentally follow lines of weakness within the shell, and therefore it appears that the foraminifer's cytoplasm can control both the extent and direction of the dissolution process. The thick resin cross-sections of *C. refulgens* attached to the valve surface revealed many visible canals extending from the base of the pit through most of the calcite layers perpendicular to the plane of the laminae (Figs. 17, 18). However as a

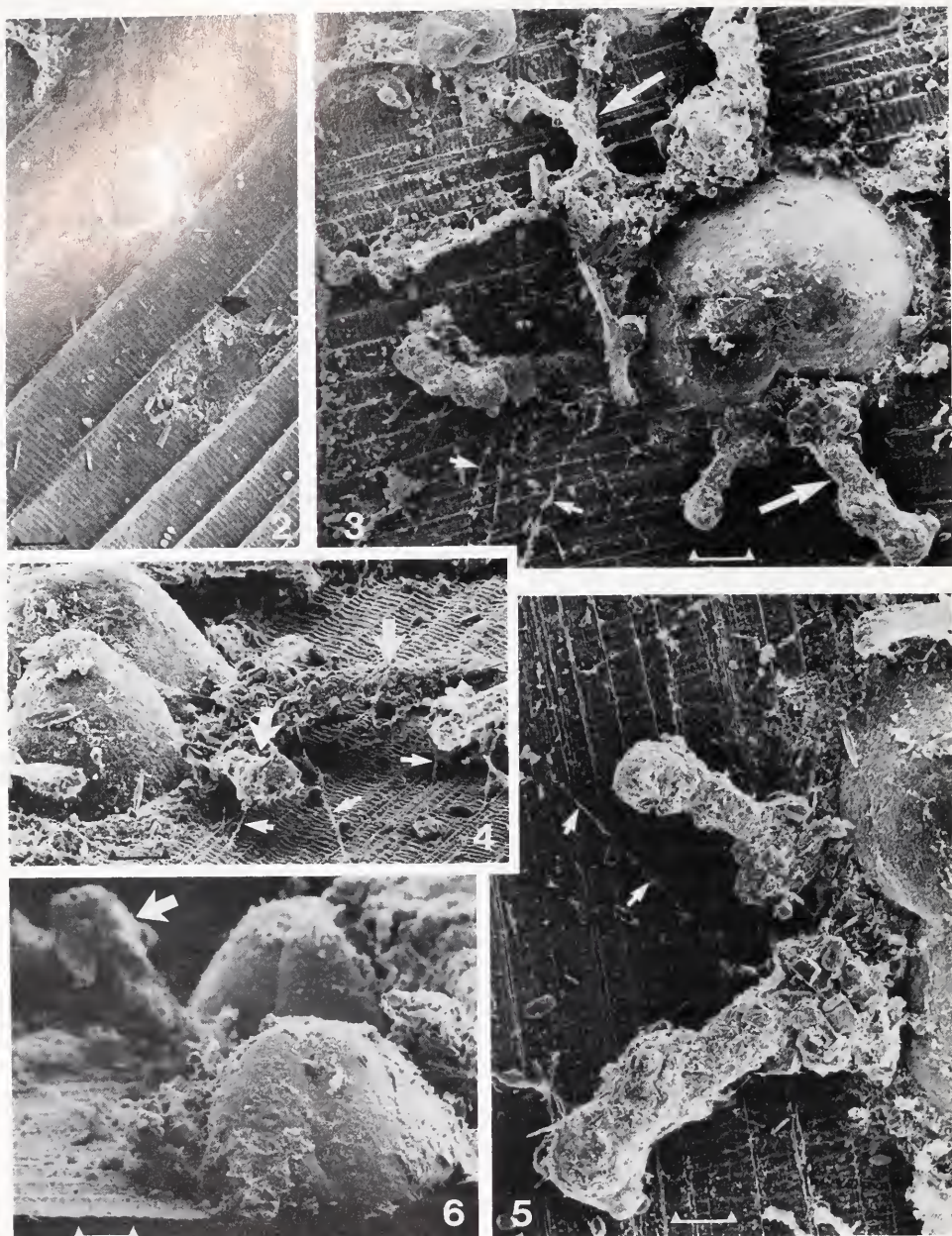


FIGURE 2. Juvenile *Cibicides refulgens* attached to surface of *A. colbecki* dorsal valve. A rudimentary peripheral agglutinated tube has been built (white arrow). Vertical tubes are not present. Two juveniles have been removed to show the shallow surface etching of the shell (black arrows). Scale bar = 163 μm .

FIGURE 3. Plan view of an attached adult with a well developed agglutinated tube system (large arrows) and net of pseudopodia on the scallop shell surface (small arrows). Scale bar = 200 μm .

FIGURE 4. Oblique view of specimen in Figure 3. Pseudopodia (small arrows) can be seen traversing the space between the agglutinated tubes (large arrows) and the substrate. Scale bar = 143 μm .

FIGURE 5. Detail of Figure 3 showing composition of agglutinated tubes and the presence of a fine pseudopod (arrows) radiating away from the foraminifer, across the shell surface. Scale bar = 102 μm .

FIGURE 6. Oblique view of two attached adult *Cibicides refulgens*. An agglutinated tube can be seen clearly raised away from the scallop shell surface (arrow). Scale bar = 154 μm .

result of the curvature of the canals, and the limited depth of field, it was not possible to photograph a single element traversing the complete shell thickness without interruption. The canal walls are significantly smoother than the adjacent calcite exposed at the fracture zone (Figs. 16, 22), but there is no evidence of an actively secreted lining.

The inner valve surface is generally lined with overlapping, angular, tile-like calcite crystals (Fig. 23), between which are many naturally occurring pores leading to the lamina beneath (Fig. 24). Upon careful scrutiny of this inner layer in the SEM, circular areas (approximately 15–50 μm in diameter) with significantly enlarged pores (Fig. 23) are evident marking the area above which an individual foraminiferan is attached on the outer valve surface. After etching for 5 to 10 minutes with 0.1 N HCl, the outermost calcite layer is removed and those salient markings are revealed more clearly (Figs. 19, 20). High magnification detail shows them to be closely spaced canal openings (Fig. 21), and there remains little doubt that these openings are continuous with the canals which originate in the surface pit, and penetrate deep into the bivalve's shell.

Examination of the exposed face of an adult *C. refulgens* detached from the substrate (Fig. 25, 26) reveals that the spiral face is not adhered to the shell material over its complete area due to a pattern of grooves radiating from the primary aperture to the peripheral test margins. The roof of each groove is the spiral test face, and the floor is formed by the etched shell material of the pit floor. Typically, four to five such grooves of approximately 30 to 50 μm width connect areas immediately adjacent to the primary aperture (Figs. 25, 29) with those more remotely situated on the opposite test margins. Etched bivalve shell which forms the base of the grooves often exhibits a pattern of fine channels (5–10 μm wide) running parallel with the main trend of the groove (Fig. 30), giving the impression of the streamlines oriented with the direction of the main cytoplasmic flow within the grooves. In addition, the lumina of the radial grooves are continuous with that of the peripheral tube encircling the test at its point of contact with the substrate (Figs. 25, 26).

Cibicides refulgens secondarily forms an elaborate agglutinated tube system around, and extending from, its attached test. The agglutinated tube system typically is comprised of: (1) a peripheral tube encircling most, if not all, of the lateral test margin at the point of contact between it and the substrate (Figs. 2, 3, 25, 26), and (2) radial tubes attached to the substrate and test, extending over the shell surface and vertically away from it (Figs. 1b, 3, 4, 27). These tubes often form several branches (Fig. 3).

All radial tubes originate from the peripheral tube, either dividing from it without any observable thickening, or arising from a distinct node at a particular point. Tubes extending horizontally along the shell surface and vertically into the overlying water may branch from the same nodes. There is no obvious organization of the branching patterns of *C. refulgens*. Typically, *C. refulgens* has from 1 to 6 ($x = 3$, $n = 50$) agglutinated tubes extending vertically up to 5 millimeters ($x = 2.5$, $n = 50$) from their points of origin at the peripheral tube. Vertical tubes may exhibit up to three orders of branching, and tube diameter does not vary consistently with length or distance from the test/substrate; thickenings or nodes can occur at any point along a tube. The interior tube surface is smoother than the outer surface (Fig. 26), and in freeze dried specimens it is partially covered with a layer of cytoplasm.

Intact tubes, when viewed in the SEM, do not show clearly defined apertures; openings are inferred by the presence of pseudopodia which extend from many points along the tubes to either the shell surface, test surface, or other tubes. Apparent aper-

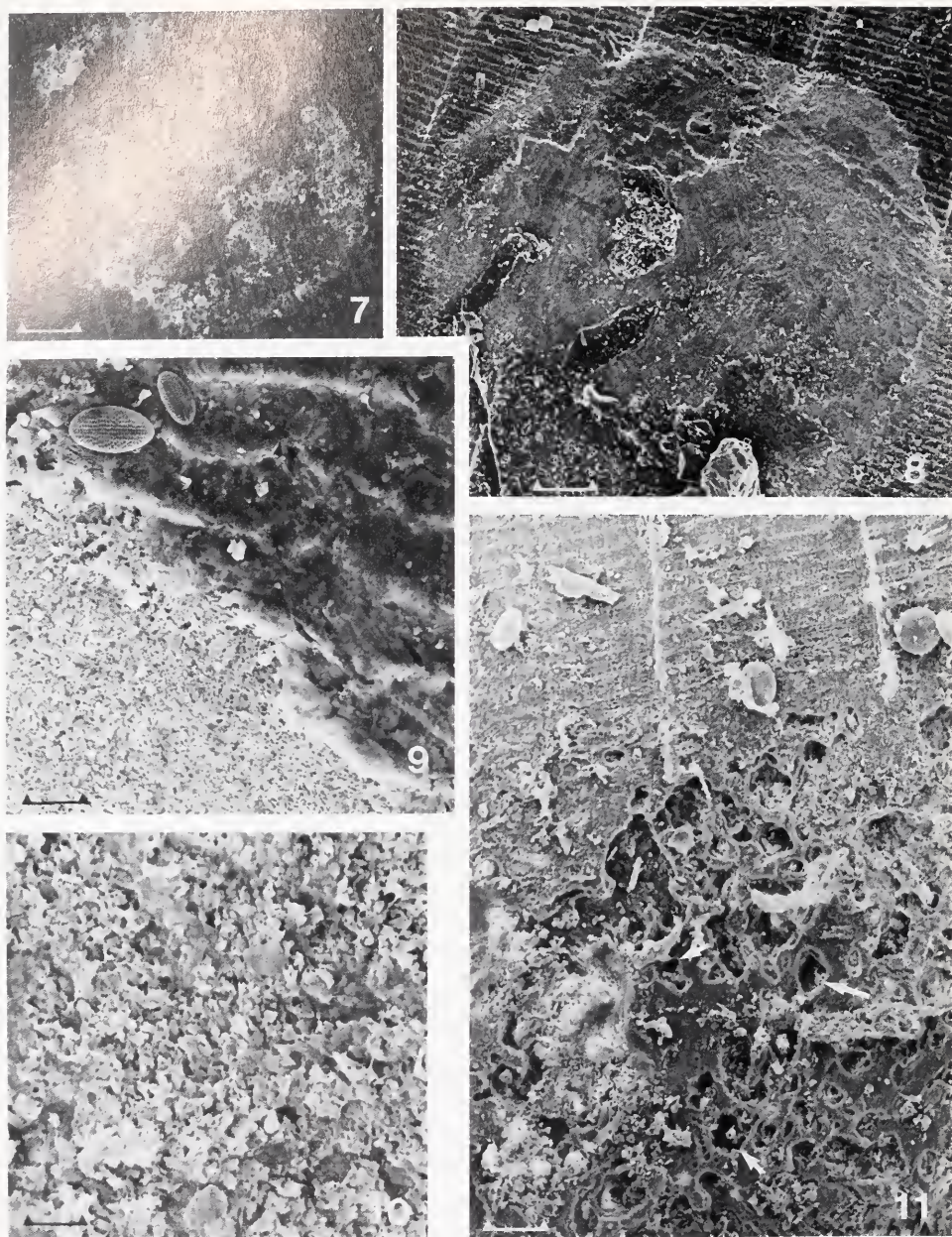


FIGURE 7. Substrate markings caused by an adult *Cibicides refulgens* after three months of attachment. There is no visible etching of the shell surface, but adhesion was great enough to break away some test material upon removal of the foraminifer. Scale bar = 200 μm .

FIGURE 8. 'Early stages' of substrate pitting. The striae of the scallop shell have been removed, and from two to three laminae have been eroded. There is no evidence of boring to form canals. Scale bar = 110 μm .

FIGURE 9. Detail of peripheral region of an early stage pit. The surface lamina has been etched away (lower left) and the calcite beneath has been partially eroded to form fine, angular granules. Scale bar = 11 μm .

tures such as that shown in Figure 31 are caused by tube breakage during collecting or transport of the scallops.

Removal of specimens from the water causes the vertical tubes to collapse against and adhere to the substrate, forming what then appears to be a system of surface tubes which resemble polychaete worm tubes. However, cytologically fixed and freeze dried tubes are able to partially support themselves thereby almost maintaining their natural positions (Figs. 4–6), and thus facilitating examination in the SEM.

The walls of all tubes are clearly agglutinated and comprise fine (silt- and clay-sized) mineral particles, diatom frustules, fine organic detritus, and occasional sponge spicules (Figs. 5, 26–28, 31). The cementing material is not clearly distinguished from the agglutinated particles and does not cover their outer surfaces. Wall thickness varies considerably but is generally from one to four particles thick with no evidence of layering or selection of specific particle size for construction. Particle recruitment by foraminiferal cytoplasm during tube construction seems to be dependent on the availability of sedimentary material on the scallop shell surface. Similarly, the incorporation of specific diatom frustules into the tubes is related to the dominant flora growing upon the scallop shell, and perhaps, the diet of the foraminifer.

Extrathalamous cytoplasm and pseudopodia were studied for gross morphology using both living and freeze dried specimens attached to pectin shells. An extensive pseudopodial net was observed spread over most of the shell surface in areas densely populated by *C. refulgens* (Fig. 32). The dorsal test surface of *C. refulgens* usually is partially covered with cytoplasm in the form of tangled strands (Figs. 32, 33). From this, randomly branching and anastomosing networks of pseudopodia emanate and connect with neighboring tests, agglutinated tubes, and/or clumps of algal or detrital material. Trunk pseudopods are usually found closer to the substrate, originating from peripheral or vertical agglutinated tubes, and traversing portions of the shell while remaining suspended above it (Figs. 3, 4, 5). Fine pseudopodial elements branch at apparently random points along trunk pseudopods and connect with others nearby (Fig. 34), or attach to the substrate beneath. These elements often merge with a finer net system attached to the substrate at raised points such as striae, and spread over most of the shell surface in the vicinity of the adult *C. refulgens* (Fig. 35). The rectilinearity and patterning of elements forming the fine nets and the limited extent of sagging when they are bearing dense mineral particles is indicative of tension within the system. The larger trunk pseudopods often visibly sag when crossing spaces between neighboring foraminifers and clumps of detrital material.

Vertical agglutinated tubes also give rise to trunk pseudopods and finer branching elements suspended freely in the water space immediately surrounding and above the tubes. Relative movement of the water in this space causes the pseudopods to bend and wave freely, demonstrating extreme flexibility in response to water movement.

All of the pseudopodia have a sticky quality when touched with single hair brushes or steel micropobes; once adhered they stretch considerably under tension before breaking. Diatoms, sedimentary particles, and organic debris are commonly observed attached to pseudopodia (Figs. 36, 37), and large clumps of detrital material were

FIGURE 10. Detail of pit base in Figure 9. Note the fine 'pores' visible between the angular calcite granules. Scale bar = 2.8 μm .

FIGURE 11. A well developed substrate pit (bottom half of micrograph) with characteristic deep borings visible (arrows) penetrating several laminae of the scallop shell. Scale bar = 62 μm .

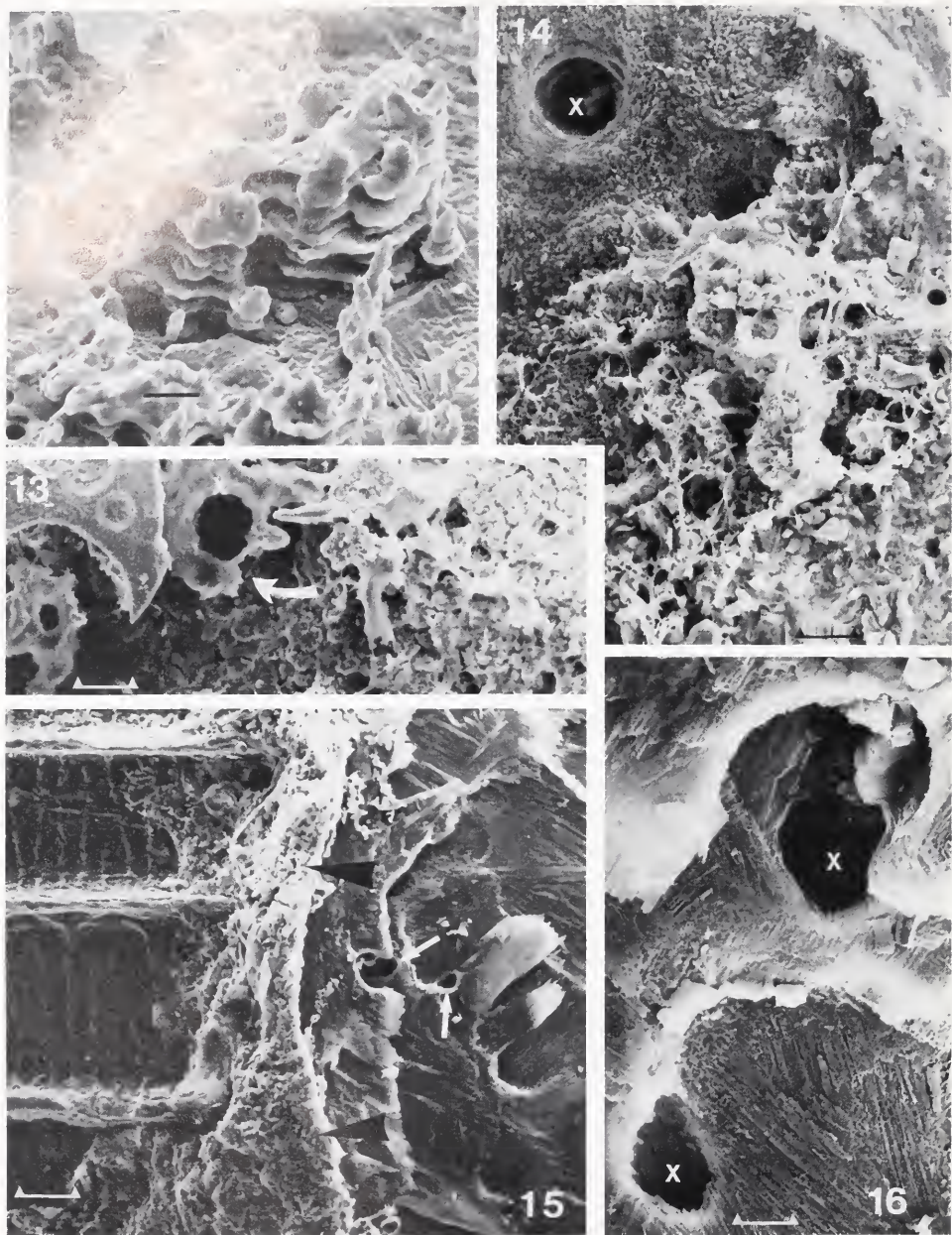


FIGURE 12. A resin cast of the central portion of a substrate pit formed by an adult *Cibicides refulgens*; the raised central area represents channels within the scallop shell which were originally occupied by foraminiferal cytoplasm. Scale bar = 20 μ m.

FIGURE 13. Irregular etching of calcite at the pit edge. A bored hole in upper calcite layer (X) has been undercut by subsequent dissolution of lower layers (arrow). Scale bar = 31 μ m.

FIGURE 14. Peripheral area of a deep substrate pit showing transition from scallop shell surface (bottom right) to extensively etched pit base (top left) and a circular vertical boring (X). Scale bar = 18 μ m.

FIGURE 15. Transition from normal scallop shell surface (left) to a deep pit (right) eroded by a large adult *Cibicides refulgens*. Removal of the foraminifer has torn away the uppermost calcite layer, exposing

often observed suspended above the substrate within pseudopodial nets. Such inclusions may be partially engulfed by cytoplasm and/or suspended by a pattern of reticular 'subnets' formed between main pseudopodial elements (Fig. 36).

Figure 40 presents the results of three experiments to further examine the sources of particulate organic material used as a nutrient source by *Cibicides refulgens*. Though patchy in distribution, benthic diatoms (primarily *Cocconeis* sp. approximately $15\ \mu\text{m}$ in length) represent a potentially significant resource to grazing organisms living on the surface of the bivalve. Time course studies monitoring the consumption of radio-labeled benthic algae by *C. refulgens* demonstrated average grazing rates of $54.5\ \text{diatoms}\ \text{mg}^{-1}\ \text{h}^{-1}$ ($n = 60$, $\text{min} = 14$, $\text{max} = 1030$). The relatively large differences in uptake rate can be accounted for by the proximity of the foraminifer's attachment site to benthic diatoms on the surface of the bivalve shell. Two other experiments examined the rate of capture of suspended bacteria and diatoms. These experiments were additionally designed to determine the relative importance of the agglutinated tubes in suspension feeding. In both of these experiments half of the attached foraminifers were cleaned of all agglutinated tubes to evaluate the importance of these structures to suspension feeding efficiency. While suspended cultures of *Nitzschia cylindricus* ($5\text{--}10\ \mu\text{m}$ at concentrations of $8 \times 10^4\ \text{cells}\ \text{ml}^{-1}$) were taken at rates of $153\ \text{cells}\ \text{mg}^{-1}\ \text{h}^{-1}$ ($n = 15$, $\text{min.} = 82$, $\text{max.} = 284$) by foraminifers with their arborescent agglutinated tube structures intact, those without this superstructure averaged rates of only $62\ \text{cells}\ \text{mg}^{-1}\ \text{h}^{-1}$ ($n = 15$, $\text{min.} = 32$, $\text{max.} = 141$). Similarly, suspended bacteria (unidentified gram negative rods $1.2 \times 2\ \mu\text{m}$ at $2 \times 10^6\ \text{cells}\ \text{ml}^{-1}$) were consumed in greater numbers by *C. refulgens* with agglutinated tubes ($x = 4.2 \times 10^2\ \text{cells}\ \text{mg}^{-1}\ \text{h}^{-1}$, $n = 15$, $\text{min} = 1.1 \times 10^2$, $\text{max.} = 6.7 \times 10^2$) than those without agglutinated tubes ($x = 69\ \text{cells}\ \text{mg}^{-1}\ \text{h}^{-1}$, $n = 15$, $\text{min.} = 21$, $\text{max.} = 1.7 \times 10^2$).

The discovery of pronounced etching channels penetrating through the shell clearly placed foraminiferal cytoplasm in contact with the nutrient-rich extrapallial space formed between the mantle and the inner surface of the shell, and suggested a parasitic relationship. Our studies using radio-labeled amino acids demonstrated the $0.5\text{--}0.7\ \text{mm}$ thick unetched bivalve shell is not permeable to free amino acids. However, when the inner surface of the bivalve shell opposite attached *C. refulgens* was bathed with radio-labeled amino acids ($100\ \mu\text{M}$), the foraminifers consistently became radioactive within a few hours. These experiments were duplicated with heat (30°C for $30\ \text{min}$) killed *C. refulgens* and no radioactivity was detected.

Figure 39 presents the uptake of uniformly ^{14}C labeled mixture of amino acids from seawater at various concentrations. This curve is clearly hyperbolic and suggests that the transport system for amino acids in *Cibicides refulgens* can be described by the Michaelis-Menten equation. The data have therefore been analyzed by a Hanes-Woolf plot (where substrate concentration divided by rate of influx is plotted against substrate concentration). As shown in Figure 40, J_{max} is $3.59 \times 10^{-3}\ \mu\text{moles}\ \text{mg}^{-1}$ (wet weight of protoplasm) h^{-1} and the K_t (substrate concentration at which the rate of uptake = $J_{\text{max}}/2$) is $10.43\ \mu\text{M}$.

Analysis of the fluid filling the extrapallial cavity were conducted using high pres-

unetched laminae beneath and canals penetrating deeper into the shell material (arrows). Part of the agglutinated peripheral tube remains secured to the shell surface (large arrowheads). Scale bar = $36\ \mu\text{m}$.

FIGURE 16. Detail of Figure 15 demonstrating the distinct canal borings (X) in shell material beneath the attached foraminifer. Scale bar = $6.7\ \mu\text{m}$.

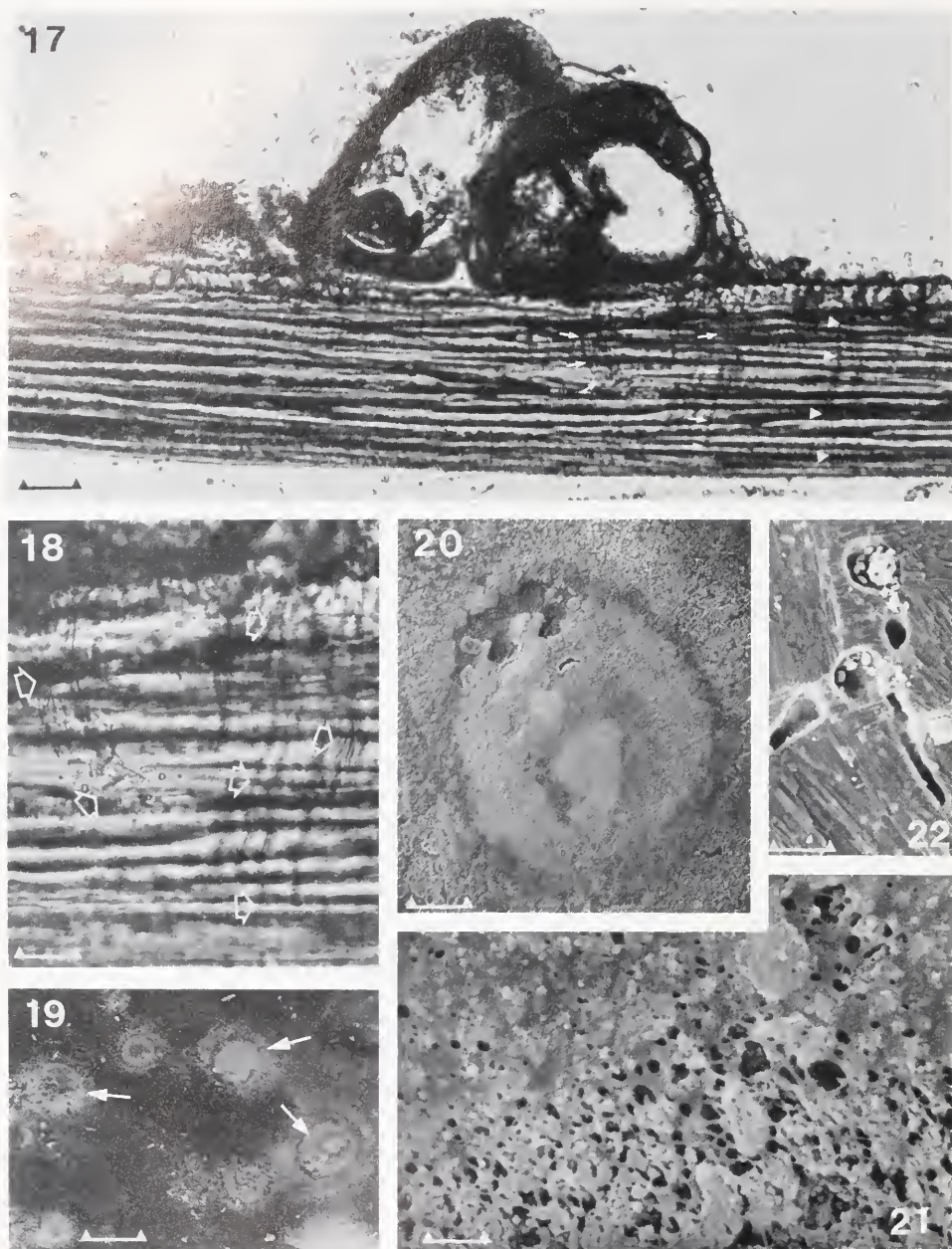


FIGURE 17. Thick cross section through a resin-embedded adult *Cibicides refulgens* attached to *Adamussium colbecki*. Groups of canals are discernible originating from the base of the pit and passing through most of the shell thickness (arrows). Scale bar = 200 μm .

FIGURE 18. Detail of Figure 17 showing the canals to be continuous through to the inner-most laminae of the scallop shell (arrows). Scale bar = 480 μm .

FIGURE 19. Acid etched inner shell surface with foraminifers attached to the opposing face. Each mark (arrows) corresponds to groups of canals penetrating the shell from the surface pits above. Scale bar = 250 μm .

sure liquid chromatography. The results (Table I) revealed concentrations of 2527 μM (2.527 mM) free amino acids with extremely high concentrations of glycine (2066.3 μM).

DISCUSSION

The rarity of loosely attached or 'roaming' *C. refulgens* on the surfaces of scallop shells strongly suggests that the sessile habit is preferred by this species. The poorly eroded pits beneath juveniles and the extensive pits associated with adults, leads to the assumption that pitting progresses with growth at least until the adult stage is reached (data on the life span of *C. refulgens* are unavailable). Specimens experimentally placed on a previously unmarked scallop shell became firmly attached to the substrate and began to construct agglutinated tubes. However, the lack of significant etching after three months raises several interesting questions: is this the typical rate of etching which would be observed by those specimens attached to live *A. colbecki*? Alternatively, is it significantly lower in response to the absence of specific cues? We have demonstrated that amino acids do not normally leach through the shell material, thus it seems unlikely that this would act as a cue to initiate excavation; cues could conceivably come from a variety of stimuli, such as rates of sediment accumulation on the shell surface, the presence or absence of organic materials from scallop excretion, and the presence/absence of water movements over the shell surface. Extensive further studies are required to investigate the role (if any) of environmental cues in initiating substrate erosion by *C. refulgens*.

Parasitism

Our investigations have shown that 50% of attached *C. refulgens* significantly erode the surface of the scallop's shell and excavate channels to the extrapallial cavity. Though the scallop shell normally is not permeable to dissolved amino acids, radiolabeled studies have consistently shown uptake of amino acids by attached foraminifers. This uptake could only have been mediated by pseudopodia penetrating through the shell. The nutritional significance of dissolved amino acids to several marine invertebrate species has been discussed by other workers (Southward and Southward, 1972; Stephens, 1981) including foraminifera (DeLaca *et al.*, 1981; DeLaca, 1982). *Cibicides refulgens* has the ability to absorb free amino acids at relatively low substrate concentrations ($K_t = 10.43 \mu M$). However, concentrations of free amino acids within the extrapallial space are more than two hundred times higher (2527 μM , [2.527 mM]) than the half saturation concentration (K_t). Therefore, a logical assumption that the foraminifer has little difficulty realizing its maximal rate of influx ($J_{max} = 3.59 \times 10^{-3} \mu g, mg^{-1}, h^{-1}$) from the scallop, and presumably this source of material would be available to the foraminifer year-round.

A wide range of associations between individuals of different species in which one

FIGURE 20. Detail of Figure 19 showing salient features directly beneath a substrate pit on the opposite shell surface. Scale bar = 60 μm .

FIGURE 21. Detail of Figure 20 showing fine perforations present in laminae exposed by etching with HCl. Scale bar = 0.9 μm .

FIGURE 22. Detail of canals exposed during fracture of the shell through a pit. These canals are approximately midway through the dorsal shell material and contain precipitated material, most probably cytoplasm. Scale bar = 23 μm .

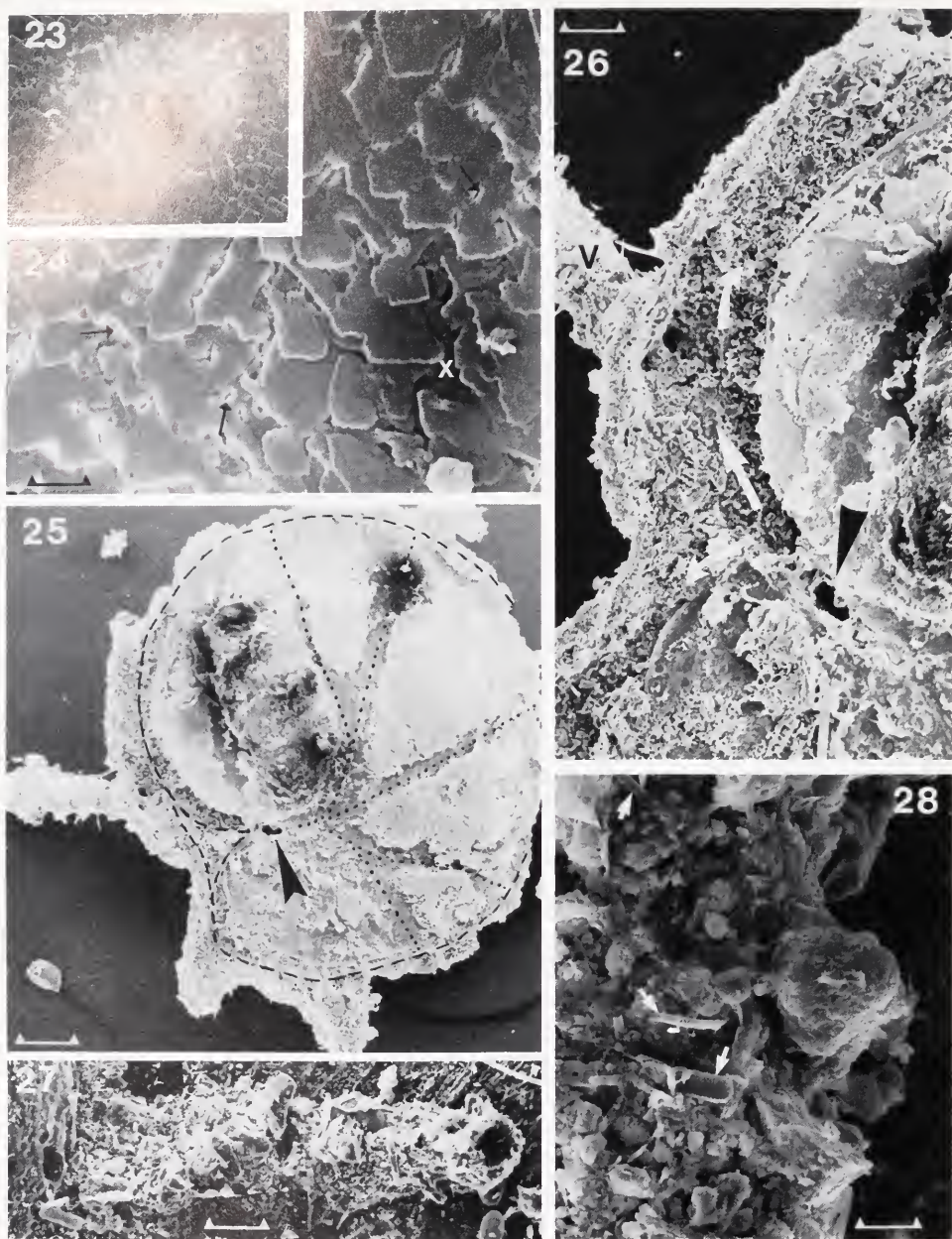


FIGURE 23. Inner surface of dorsal scallop valve showing marking which is often observed when the opposing surface is heavily colonized with *Cibicides refulgens*. Scale bar = 6.2 μm .

FIGURE 24. Detail of Figure 23 demonstrating enlarged 'pore' between calcite plates (X) and numerous 'micropores' located peripherally (arrows). Scale bar = 1.0 μm .

FIGURE 25. Umbilical view of an adult *Cibicides refulgens* removed from the valve surface. The primary aperture (arrow head) opens into (a) the lumen of the peripheral agglutinated tube (dashed line) and (b) grooves created between the umbilical face and the base of the pit (dotted lines). Scale bar = 150 μm .

or both derive benefit from the other have been described in the literature. They range from being obligate to being facultative (each partner being able to live without the involvement of the other), and the grades of association within this range often are not distinct. For convenience the relationships are frequently termed commensal and parasitic. By definition, a parasite always lives to the detriment of its host. Parasitic life styles are frequently specialized and lead to development of morphological as well as physiological adaptations which ensure efficiency. The relationship between *C. refulgens* and *A. colbecki* is very similar to that described by Todd (1965) for *Rosalina carnivora* and *Lima angolensis*. Unlike Todd's work however, the present study presents unambiguous evidence that *C. refulgens* does derive nourishment from the mantle of its host. Whether the cumulative affects of approximately 900 attached *C. refulgens* (~400 [45%] of which may have created channels through the shell) have a detrimental affect on the bivalve in this marginal environment remains unknown, but seems likely.

Grazing

Morphological test elaboration in the form of a constructed horizontal tube systems on the scallop shell surface effectively increases the distance that pseudopodia can gather food without severely increasing risk of cytoplasmic loss to predation or other causes. For example, we have observed tanaid crustaceans living in tubes on the scallop shell and feeding on unprotected cytoplasm of extended pseudopodia from *C. refulgens*. Of course, cytoplasm within the agglutinated tubes of the foraminifer is contiguous with cytoplasm in the lumen of the last formed chamber and thus the tubes are regarded as an extension of that chamber. Whereas most calcareous foraminifera are compelled to withdraw all extrathalamous cytoplasm into the test when unfavorable conditions or predators are encountered, *C. refulgens* individuals need only withdraw pseudopodia into the agglutinated tubes for protection. Thus the total volume of cytoplasm deployed, and therefore the total area grazed, is vastly increased without much risk of cytoplasmic loss. Most calcareous foraminifera use only the cytoplasm present in the last formed, penultimate and sometimes the antepenultimate chamber for pseudopodia and extrathalamous cytoplasmic activity (Anderson and Be, 1978; Anderson, 1983; Alexander and Banner, 1984; Alexander, 1985), and therefore may gather food at limited distances from the test without considerable risk to cytoplasm.

Adamussium colbecki shells typically are colonized by benthic diatoms and bacteria, but their concentrations, diversity, and percentage of surface coverage, however, vary from specimen to specimen. This heterogeneity is typical in New Harbor both spatially and temporally on large and small scales, and organic productivity in this portion of McMurdo Sound is extremely seasonal; our observations indicate that pronounced shallow-water productivity may be limited to as little as 2½ months. (DeLaca, unpub. data). Although approximately six months of continuous sunlight

FIGURE 26. Detail of Figure 25, demonstrating continuity of the primary aperture (arrow head), with the lumen of the peripheral agglutinated tube (arrows) and that of a vertical agglutinated tube (V). Scale bar = 67 μ m.

FIGURE 27. Surface morphology of a typical agglutinated tube (in this case, radial and in contact with the substrate). Scale bar = 67 μ m.

FIGURE 28. Detail of typical agglutinated material forming tube walls. Arrows = diatom frustules. Scale bar = 12 μ m.

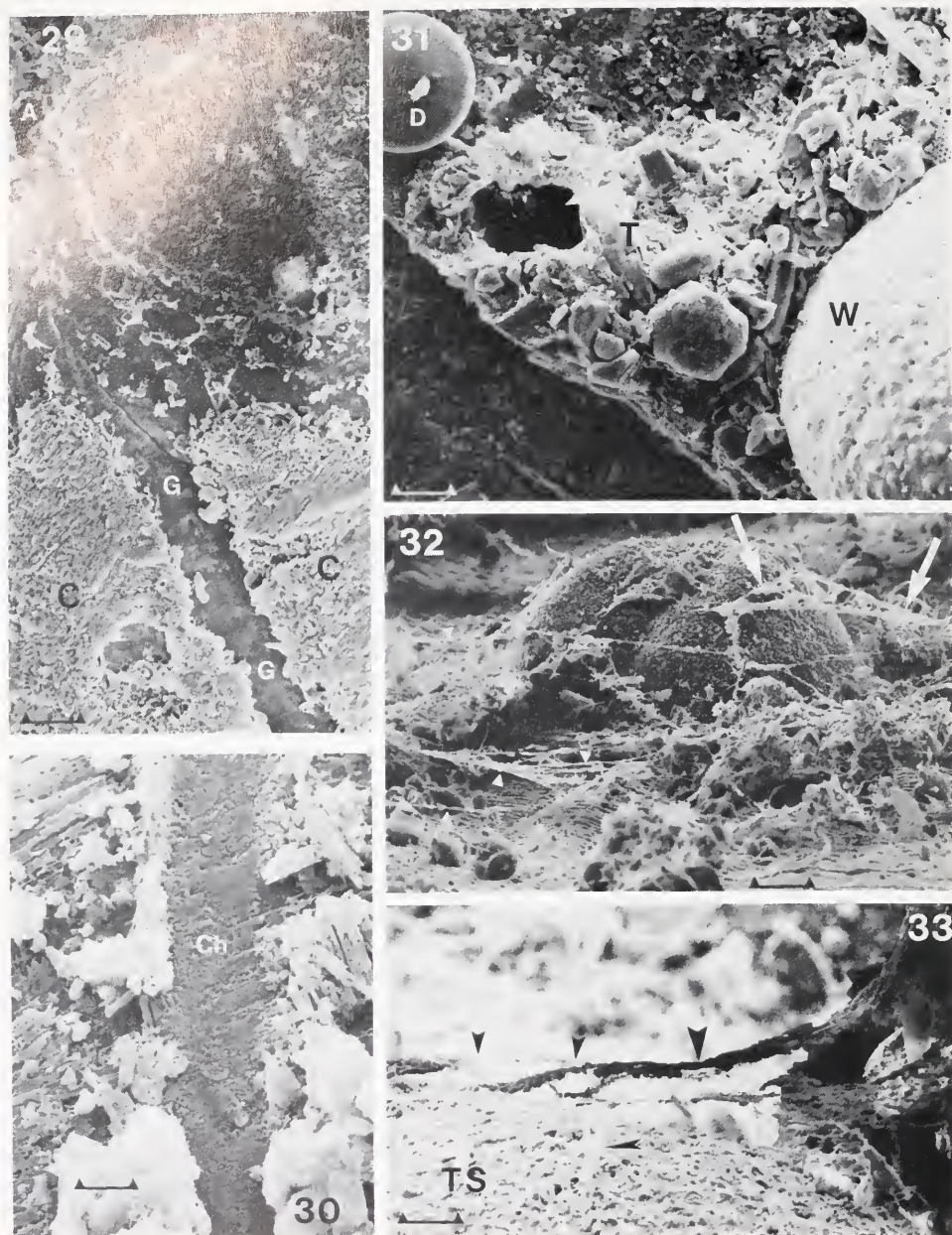


FIGURE 29. Detail of Figure 25 showing a radial umbilical 'groove' (G) which exists between the substrate surface in the pit and the umbilical test wall. A = primary aperture; C = Calcite broken away from pit base. Scale bar = 48 μ m.

FIGURE 30. A typical eroded 'channel' (Ch) commonly observed on calcite which forms the base of umbilical 'grooves' in attachment pits. Scale bar = 4.2 μ m.

FIGURE 31. Detail of agglutinated tubes (T) formed by juvenile *Cibicides refulgens* in Figure 2. D = Diatom; W = test wall of last formed chamber. Scale bar = 18 μ m.

FIGURE 32. Oblique view of adult *Cibicides refulgens* attached to dorsal scallop valve. An extensive net of pseudopodia (arrow heads) is visible over the substrate and extending from the dorsal test surface (arrows). Scale bar = 83 μ m.

is available annually, the combination of low angle of incident radiation, sea ice, and snow cover reduces the period of primary productivity further (see Dayton and Oliver, 1977).

Benthic diatoms on the scallop shell surface, as well as amorphous organic material and sediment, were attached to and transported by pseudopodia. Our experiments using *in situ* ^{14}C -labeled attached, and motile benthic diatoms, demonstrate that the foraminifers graze upon the naturally occurring 'lawns' of algae, and that relatively high numbers ($x = 54$ diatoms $\text{mg}^{-1} \text{h}^{-1}$) are consumed. Through the approximation of biomass and conversion to carbon content (Strathmann, 1967), it is estimated that if rate of harvest remained constant, those benthic diatoms would have contributed approximately $1 \times 10^{-3} \mu\text{g C mg}^{-1} \text{h}^{-1}$. That value is approximately one-half the amount of carbon obtained through the uptake of dissolved amino acids; thus grazing microorganisms from the surface of the scallop shell appears to be an important factor in *C. refulgens* nutrition. The discorbid foraminifer, *Rosalina globularis*, also forms deep pits in its preferred substrate (DeLaca and Lipps, 1972), and grazes upon algae in the immediate vicinity; however, in conditions of low food concentration it roams in search of algae (Sliter, 1965). In view of the patchy distribution of algae on *A. colbecki*, the selection of a sedentary habit by *C. refulgens* seems to have potentially reduced its grazing abilities. This disadvantage is more than compensated for by the permanent, or semi-permanent attachment between the scallop shell and the foraminifer, which virtually eliminates the risk of being swept off the shell during swimming movements of the scallop, and enables further morphological and physiological adaptations to the epizoid habit.

The radial grooves existing between the spiral side of the foraminifer's test and the surface of the pits increases the efficiency of this greater cytoplasmic volume through provision of a short line of communication between the primary aperture and the lumen of the peripheral tube on the opposite test side; this facilitates rapid exchange of cytoplasmic organelles and inclusions such as mitochondria, phagocytosed material, and energy substrates between the most distal pseudopodia and the intrathalamic cytoplasm. These internal-external lines of communication between deeply situated cytoplasm and the external milieu, are considered important in foraminiferan cell systems (Brasier, 1982).

Suspension feeding

Using the vertical agglutinated tubes as conduits for streaming pseudopodia and as anchors for pseudopodial nets, *C. refulgens* exploits suspension feeding as a third trophic mechanism. Figure 38 depicts the most typical arrangement of pseudopodia in an undisturbed living specimen; free pseudopodia are not rigid structures, but yield to water movement. Pseudopodial nets are randomly arranged and thus form a wide range of mesh sizes. Construction of the nets is initiated by the extension of pseudopodia from apertures along the vertical tubes, followed by contact with other tubes or nearby structures, and elaboration through bi-directional cytoplasmic flow. While unsupported pseudopodia of *C. refulgens* also have been seen projecting into the water, the construction of an agglutinated tube system provides scaffolding for further suspended pseudopodia within the water column, as well as a reservoir of protected

FIGURE 33. Dorsal test surface (TS) of an adult *Cibicides refulgens* showing cytoplasmic strands (arrow heads) reaching to adjacent detrital material (far right). Scale bar = $3.2 \mu\text{m}$.

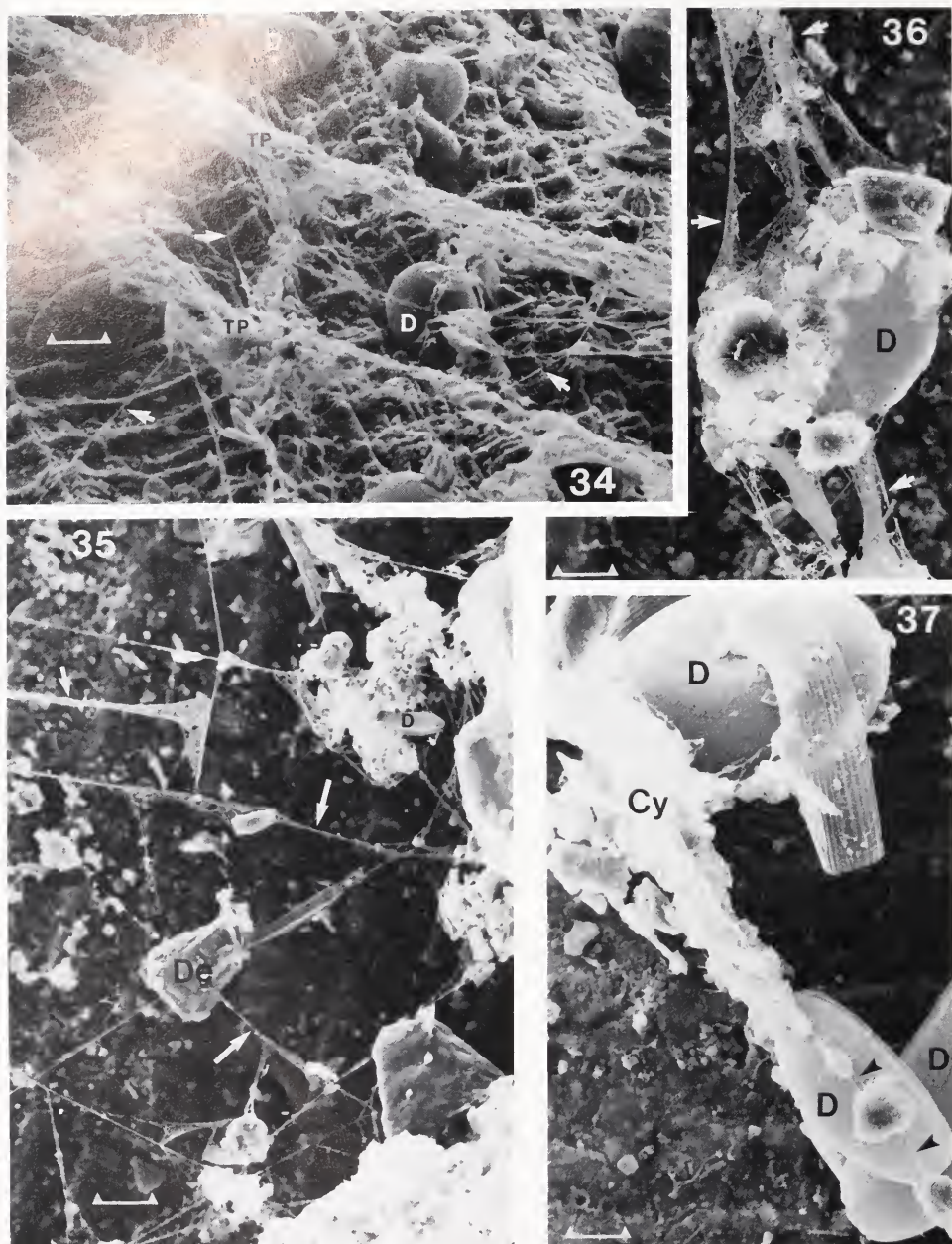


FIGURE 34. Trunk pseudopodia (TP) crossing the scallop shell surface and radiating away from a large adult *Cebicides refulgens*. Fine pseudopodia can be seen branching from the main trunk and attaching to the substrate (arrows). D = diatoms. Scale bar = 16.6 μm .

FIGURE 35. Finely branching pseudopodia (arrows) forming a net above the substrate. Detrital material (De) and diatoms (D) are entrained by pseudopodia. Scale bar = 17.2 μm .

FIGURE 36. Diatom (D) and attached detritus suspended above substrate by a fine anastomosing pseudopodia (arrows). Scale bar = 6.9 μm .

FIGURE 37. Cytoplasm (Cy) of pseudopod which has adhered to several diatoms (D) on the substrate surface. Note fine cytoplasmic threads (arrow heads). Scale bar = 8 μm .

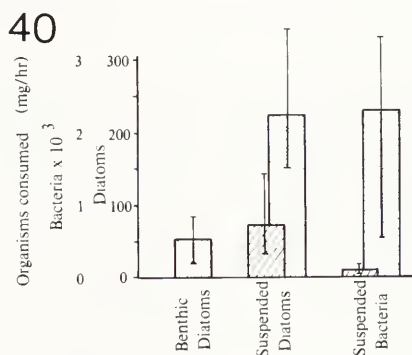
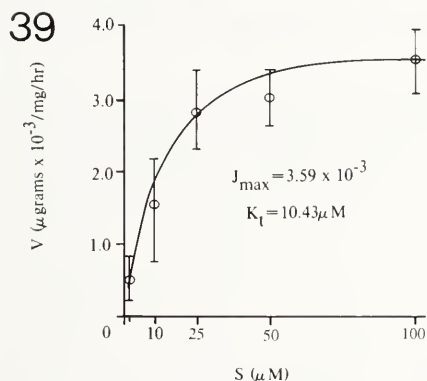
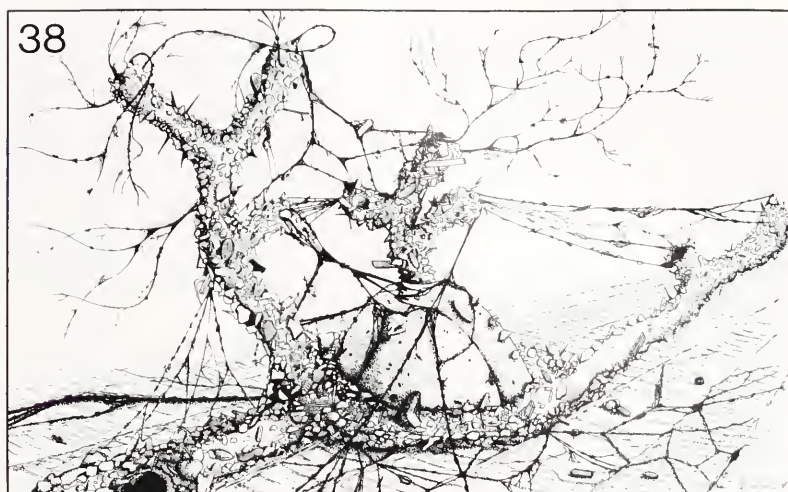


FIGURE 38. *Cibicides refulgens* attached to the shell of *Adamussium colbecki*, with pseudopodia deployed from agglutinated structures. (Not to scale.)

FIGURE 39. Velocity of uptake as a function of the concentration of dissolved amino acids in seawater. The values for $J_{\max}$ ($\mu\text{grams}/\text{mg h}$) and K_t were obtained from a Hanes-Wolff linear transformation of the data. Each point is the mean of 10 replicates; bars represent the range of measurements.

FIGURE 40. Numbers of bacteria or diatoms taken (organisms/ mg h) by *C. refulgens* through grazing (first bar) or suspension feeding. All agglutinated material was removed from half of the attached foraminifers (diagonal lines) while the remaining subpopulation was undisturbed (stippling) in order to determine if suspension feeding was facilitated by the agglutinated test extensions. Bar heights represent mean values (15 replicates for each suspension experiment and 60 replicates for the grazing experiment); error bars depict the range of values for each experiment.

cytoplasm. Agglutinated tubes radiate away from the substrate and have been measured to heights of five millimeters, and pseudopodia have been measured to extend an additional three millimeters from the tips of these tubes. The resulting canopy of branching tubes and pseudopodia potentially increases the volume of water available to suspension feeding by a factor of 10 to 20. Suspension feeding efficiency is further enhanced by the near proximity of other foraminifers and their tubes.

Our experiments using radio-labeled prey demonstrated that *C. refulgens* cap-

TABLE I

Free amino acids present in the extrapallial space of Adamussium colbecki

Amino acid	Concentration of AA in extrapallial cavity μM	Percent AA in ^{14}C protein hydrolysate*
Alanine	87.1	9.3
Arginine	165.5	6.3
Asparagine	11.0	0.0
Glycine	2066.3	4.6
Histidine	0.0	4.0
Phenylalanine	26.5	6.7
Proline	—	5.6
Serine	76.8	4.8
Tyrosine	11.8	3.6
Glutamic acid	24.1	11.8
Valine	13.1	6.8
Isoleucine	8.9	4.8
Leucine	12.7	11.8
Lysine	17.3	5.1

* An analysis given by Amersham Corporation for its product CFB.25 ([U- ^{14}C]algal protein hydrolysate).

tured both suspended diatoms and bacteria, and that capture efficiency was enhanced by agglutinated tubes by factors of 2.5 and 6.1 for diatoms and bacteria, respectively. It is relevant to note here that to facilitate the experiment, concentrations of both suspended food sources were higher than the foraminifers would experience naturally.

Several basic mechanisms of filter feeding are involved in systems such as that used by *C. refulgens*. According to Rubenstein and Koehl (1977), true sieving would not be an important contribution to particle capture in the nets of *C. refulgens* because the average mesh size far exceeds the diameter of particles most likely to be encountered in the fine detritus of New Harbor. 'Direct interception' of particles by the sticky pseudopodia would form a large part of the filtering process, as would 'motile particle deposition' of, for example, motile algae and bacteria. 'Gravitational deposition' of particles resuspended by the non-locomotory flapping motion of the scallops, is likely to provide an important source of captured material. The requirement for water movement to allow filter feeding through the pseudopodial sieve must be satisfied almost entirely by movements of scallops, either locomotory (which occurs infrequently; see Mullineaux and DeLaca, 1984), non-locomotory, or by low velocity localized turbidity currents observed by DeLaca *et al.* (1980).

How material is entrapped by pseudopodia of *C. refulgens* and transported to the agglutinated tubes or the cell body, has not been previously investigated. However, detailed information is available on the ultrastructural aspects of particle/prey entrapment and transport by pseudopodia of the Antarctic foraminifer *Astrammmina rara* (see Bowser and DeLaca 1985a, b; Bowser *et al.*, 1986), and *Allogromia* sp. (Bowser and McGee-Russell, 1982). In *Allogromia* sp. attachment is mediated by 'ultramicrospikes' and 'ultramicrowebs' which possess special adhesive properties (Fig. 3, in Bowser and McGee-Russell, 1982; McGee-Russell *et al.*, 1982). Structures almost identical to the ultramicrowebs of *Allogromia* sp. were observed in *C. refulgens*. These structures were found at the points of pseudopodial bifurcation, and in areas where particles were suspended (see Fig. 37) by the pseudopodia. That these structures sur-

vived the crude freeze-drying techniques available to us in Antarctica, without low calcium treatment and critical point drying, suggests that they may be even more extensive than our results indicate. These two taxonomically distant species appear to employ a similar mechanism for particle entrapment.

Suspension feeding has been reported for a number of benthic foraminiferal genera, most of which possess elevated stalk-like tests anchored at one end to the substrate (See Christiansen, 1971; and Lipps, 1982; 1983 for reviews); in addition, two species of the benthic rotaliid *Elphidium* have been observed with three-dimensional pseudopodial networks extending into the seawater medium, which would be efficient collectors of free-floating food particles (Jepps, 1942; Sheehan and Banner, 1972). In addition, the arborescent foraminifer *Notodendrodes antarctikos* DeLaca, living in New Harbor, captures bottom sediments which are brought into suspension by activities of larger benthic invertebrates (DeLaca *et al.*, 1980) and, as with *C. refulgens*, this specialized mode of feeding is regarded as being an adaptation to an unusual oligotrophic environment. The availability of resuspended organic material could provide a more consistent source of food during the dark austral winter when benthic diatom productivity is low, and as such would compliment nutrients obtained by other mechanisms such as the uptake of free amino acids from the sediment and surrounding seawater in the case of *N. antarctikos*, and parasitism in the case of *C. refulgens*.

The incorporation of agglutinated material into the test of calcareous foraminifera is an uncommon phenomenon which appears to be restricted to the suborder Miliolina. Within the family Miliolidae, three genera (*Sigmoilopsis*, *Ammomasilina*, and *Schlumbergerina*) reportedly have test walls composed of agglutinated material bound by a calcareous cement; the genus *Dentostomina* has an external agglutinated layer of grains (Loeblich and Tappan, 1964). Within the Nubecularidae, *Nubeculina* has much coarse agglutinated material on the exterior of its chambers, and *Nodobacularia* incorporates occasional sand grains into the test (*opera. cita.*). However, among the suborder Rotaliina, test construction involving both agglutinated and calcareous material is rare. Nyholm (1961) described a coniform stage of *Cibicides lobatulus* (which he regards as having developed from a zygote) with associated "tube-shaped structures composed of agglutinated material" extending vertically from the apex of the test, or occasionally, basally branching and leading from the aperture. Nyholm (1961) noted that an interspace of a few microns exists between the cytoplasm and the agglutinated wall of the coniform test; this may be morphologically analogous to the lumen of the circular agglutinated tube at the periphery of the test in *C. refulgens*. Indeed, regardless of which part of *C. lobatulus*' life cycle these agglutinated tubes are associated, it is apparent that they are, in some respects, structurally similar. Nyholm (1961) did not suggest a function for the agglutinated tubes which project into the water above the attached sarcod (although he states that the agglutinated coniform test determines the form of the outer calcareous chambers), but in light of our findings with *C. refulgens*, it is tempting to speculate that they are also concerned with the deployment of pseudopodia into the surrounding water as a mechanism for suspension feeding.

The taxonomic and phylogenetic significance of extensive agglutinated additions to the tests of members of the genus *Cibicides* is not understood and remains an interesting question for taxonomists.

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TIDAL HEIGHT AND GAMETOGENESIS: REPRODUCTIVE VARIATION AMONG POPULATIONS OF *GEUKENSIA DEMISSA**

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ABSTRACT

High tidal populations of the mussel *Geukensia demissa* experience reduced filter feeding time as a result of aerial exposure. This study tested the hypothesis that such populations exhibit a temporal delay in their gametogenic cycle compared to populations from the low intertidal. As predicted, quantitative estimations of gametogenic condition of mussels from 10 high tidal populations were lower than those of mussels from 11 low intertidal populations in May 1986. A two-fold difference in potential feeding time was accompanied by a delay of about two months in the reproductive activity of high tidal mussels. This study demonstrates that temporal reproductive variation among populations of *G. demissa* across the intertidal zone may be as large, or larger than variation among latitudinally separated populations of this species. Site-to-site variation in timing of reproduction within the North Inlet Estuary may also be as large as temporal latitudinal variation. Level of occurrence in the intertidal zone and hence length of submersion and potential feeding time exert profound influence in the timing of the reproductive cycle of the ribbed mussel, *Geukensia demissa*.

INTRODUCTION

Variation in the timing of gametogenesis and spawning has been documented among populations of a number of marine invertebrates (Giese and Pearse, 1974, for a review). Such variation is common among populations of bivalve molluscs (Lubet *et al.*, 1981; Newell *et al.*, 1982; Bayne and Newell, 1983), but the causes of this variability are not understood. Differences of environmental temperature (Orton, 1920; Sastry, 1970) and seasonality of abundance and composition of food (Newell *et al.*, 1982; Rodhouse *et al.*, 1984) are thought to affect the reproductive cycles of marine bivalves. Latitudinally separated populations of the same species may exhibit large differences in the timing of reproductive activities (Sastry, 1970; Lubet *et al.*, 1981; Barber and Blake, 1983; Brown, 1984), but comparable variation also may be observed among populations separated by much smaller distances (Seed and Brown, 1977; Newell *et al.*, 1982). Microgeographic variation in the timing of reproductive activities may be due to site-specific differences in food supply (Bayne and Worrall, 1980; Newell *et al.*, 1982; Worrall *et al.*, 1983; Rodhouse *et al.*, 1984). However, the causes of variation in timing and/or intensity of reproduction have been established

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Abbreviation: GVF, Gamete Volume Fraction.

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in only a few cases (Mann, 1979; Velez and Epifanio, 1981; MacDonald and Thompson, 1986).

Variation in height along the intertidal zone poses a dramatic gradient of food availability for filter feeding organisms. Since feeding can only occur during submersion, the potential feeding time is limited by the length of submersion. The effect of differences in potential feeding time on the energy balance of filter feeding animals is not clearly understood, but food availability and nutritional condition may strongly influence reproductive activity (Barber and Blake, 1983; Bayne and Newell, 1983). Therefore, differences in feeding time may have important consequences on the gametogenic cycles.

This study examines the reproductive cycles of populations of the ribbed mussel *Geukensia demissa* (Dillwyn) (Bivalvia: Mytilidae) that occupy different tidal levels in the same salt marsh habitat. Previous studies suggested that diminishing the nutritional status of bivalve molluscs may delay their gametogenic cycle (Mann, 1979; Velez and Epifanio, 1981). Therefore, the specific hypothesis that the reproductive cycle of *G. demissa* will be delayed in high intertidal mussels which experience decreased potential feeding time relative to low tidal mussels, was tested.

Although *G. demissa* is a dominant secondary producer in salt marsh ecosystems of the eastern United States (Kuenzler, 1961; Fell *et al.*, 1982; Jordan and Valiela, 1982; Bertness, 1984), information on its reproductive biology is scarce. Ribbed mussel populations from New England and Connecticut exhibit a single spawning period from June through September (Brousseau, 1982; Jordan and Valiela, 1982), whereas in North Carolina and Georgia, peak reproductive activity might occur later, and/or gametogenic activity extend longer, in the year (McDougall, 1943; Kuenzler, 1961). This study provides a quantitative comparison of the reproductive cycles of populations of *G. demissa* from South Carolina and describes temporal reproductive variation associated with level of occurrence in the intertidal zone.

MATERIALS AND METHODS

During 1983–84, monthly samples of mussels were collected from a low tidal site (Site 1, Up Clambank) and a high tidal site (Site 2, Bly Creek), less than 1 km apart, within the North Inlet Estuary, South Carolina (Fig. 1). The high-tidal site is a short-form *Spartina alterniflora* marsh habitat, which is covered by water approximately 4 hours per day. The low tidal site is an intertidal oyster (*Crassostrea virginica*) bed, where mussels occur among the oysters. This site is covered by water for about 8 hours per day. Each sample consisted of approximately 25 mussels ranging from 25 to 100 mm in shell length.

Mussels were brought to the laboratory, scrubbed clean, and their shell lengths measured. A 1-cm² section was cut from the same area of the right mantle lobe of each animal and processed as follows. Serial sections 5 microns thick were prepared from paraffin embedded tissues and stained with hematoxylin and eosin. The fractional area of the mantle section that is composed of gametes (Gamete Volume Fraction, GVF) was determined using stereology (Lowe *et al.*, 1982), and expressed as the mean GVF for each sample. GVF values were arcsine transformed to assure normal distribution. Two-way ANOVA (Sokal and Rohlf, 1981) was used to examine the effect of date and site on reproductive condition of mussels. The effects of date and sex were determined separately for each site, using two-way ANOVA. Mussels were separated into six shell length classes, and the effect of shell length on reproductive condition was determined using ANCOVA (Sokal and Rohlf, 1981). Mussel shell length was used as the covariate. Mean GVF values were detransformed for illustra-

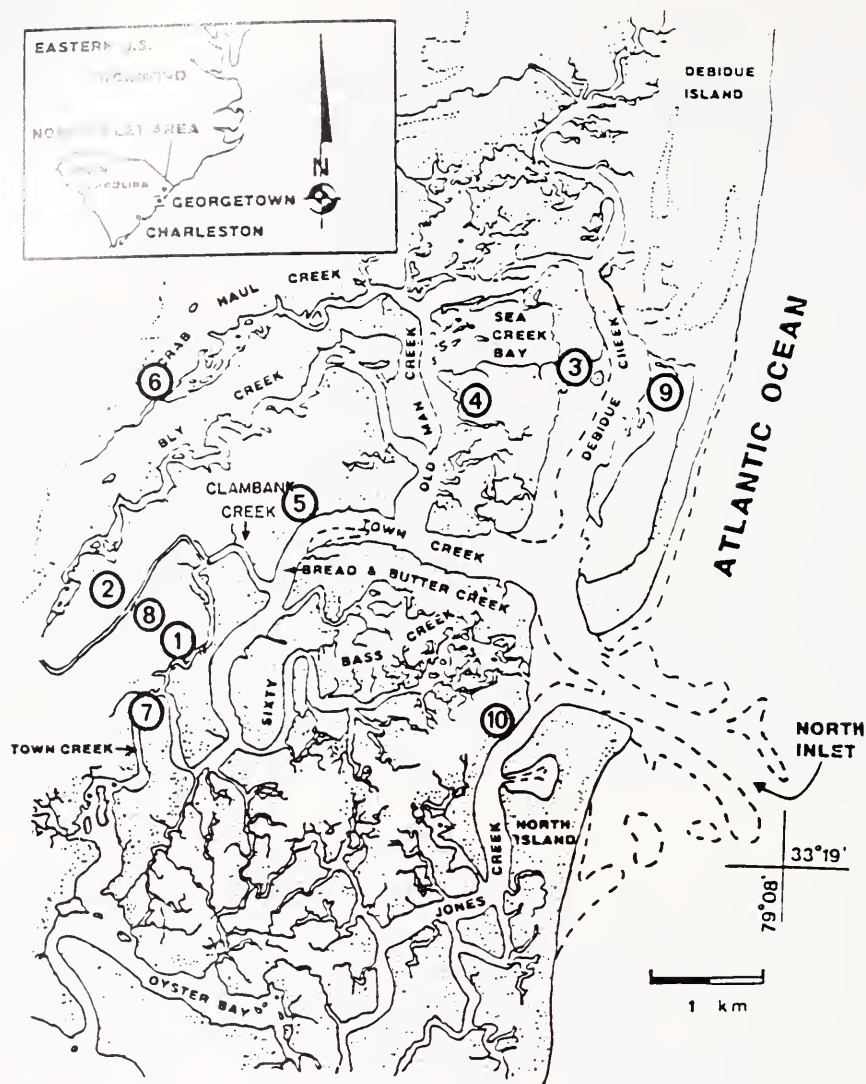


FIGURE 1. Map of the North Inlet Estuary, South Carolina, indicating the ten sites studied. 1 = Up-Clambank, 2 = Bly Creek, 3 = Debidue Creek, 4 = Old Man Creek, 5 = Town Creek, 6 = Oyster Landing, 7 = Oyster Island, 8 = Goat Island, 9 = Debidue Island, 10 = Jones Creek.

tion in figures. Data on water temperatures at sites within the North Inlet were available from the Long Term Ecological Research (LTER) program of the Baruch Institute, University of South Carolina.

RESULTS

Temporal differences in the reproductive cycles of the high and low tidal populations of *G. demissa* were observed. At the low tidal site, mussels had relatively con-

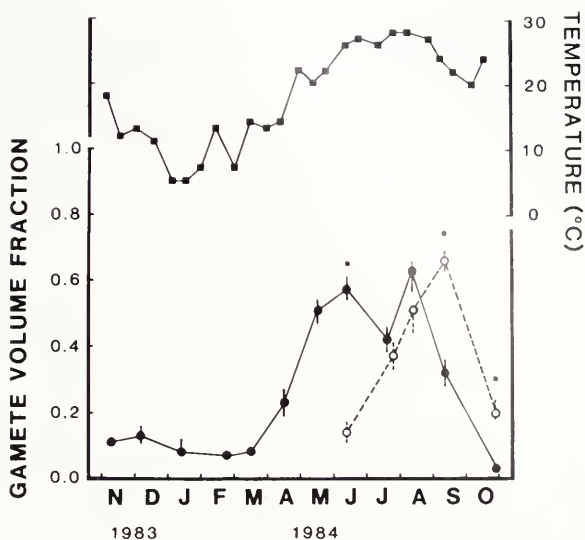


FIGURE 2. Reproductive cycles of *Geukensia demissa* during 1983–84, from a low tidal population (Site 1, Up-Clambank: ●), and a high tidal population (Site 2, Bly Creek: ○). Mean values of total GVF for approximately 25 individuals are plotted each month. Vertical lines indicate the standard error of the mean. Asterisks indicate significant differences ($P < 0.05$) between the two sites. Seasonal variation in surface water temperature (■) at Bread and Butter Creek (see Fig. 1). Each point represents a single measurement of temperature.

stant and low GVF values from November to March, after which a rapid increase of GVF occurred, reaching a peak of about 0.57 by June (Fig. 2). By late April, no animals with sexually undifferentiated gonads were found. The increase of mean GVF closely followed the pattern of increasing water temperature in North Inlet, showing a significant ($P < 0.05$) and positive correlation ($r = 0.776$). This population maintained relatively high values of GVF for about four months (May to early August). The highest GVF value was observed in August (0.65), and spawning probably took place in this month and proceeded through September. It is possible that another spawning event of lesser intensity occurred at this site in June, which is supported by the observation of a significant ($P < 0.05$, Tukey *a-posteriori* test) decline of about 20% in the mean GVF values between June and July.

At the high tidal site, mussels exhibited a different reproductive cycle (Fig. 2). Gametogenesis started later, possibly between May and June. A rapid increase of GVF occurred between June and September, reaching an apparent peak of about 0.65 in September. Spawning occurred in September, shortly after gametogenic development (Fig. 2). Since information on the reproductive condition of this population was available only from June to October, a correlation analysis between GVF and temperature is not very meaningful. However, the reproductive cycle of mussels at this site was delayed 2–3 months with respect to the pattern of increase in water temperature (Fig. 2).

The ANOVA procedure indicated temporal differences ($P < 0.05$) in the reproductive cycle of the high and low tidal populations of *Geukensia*. The pattern of gametogenic development of male and female mussels was similar in the magnitude of the GVF values attained. However, male mussels appeared to start maturing earlier than females in both populations, since the sex ratio was skewed toward males in

samples collected between November and February at the low tidal site, and in June at the high tidal site. As the gametogenic cycle proceeded, the sex ratio did not differ significantly from 1:1 in later samples from either site. No evidence of hermaphroditism was observed from 429 mussels examined. Sexually undifferentiated animals were present until April at the low tidal site, and until as late as July at the high tidal site, providing further evidence for temporal displacement of the onset of gametogenesis between the two populations. ANCOVA did not indicate a significant effect of shell length on the GVF values observed on animals from either population ($P > 0.05$).

In summary, the reproductive cycles of the two populations differed in three major aspects: timing of the onset of gametogenesis; time of occurrence of spawning; and length of time mussels remained in a mature reproductive condition prior to spawning. Interestingly, despite the above temporal differences in reproduction, the highest GVF values observed on mussels from the two sites were similar (Fig. 2).

A sampling program involving mussels from high and low tidal levels at a number of sites including the two original populations was conducted to determine whether the observed temporal differences in the reproductive cycles were restricted to the two sites studied, or whether they represented a general phenomenon among mussel populations across the intertidal zone. Since the 1983–84 samples indicated that the difference in reproductive condition was greatest at the end of the spring (Fig. 2), sampling was conducted on 24 May 1986. Based upon the results of the earlier sampling, the prediction that in May high tidal mussels should exhibit lower GVF values than mussels from the low intertidal was made. To test this hypothesis, ten sites were chosen such that low and high tidal mussel populations could be found (Fig. 1). Approximately 15 mussels, 40–90 mm long, were collected from low and high tidal levels at each site. The high tidal level was *Spartina* marsh, similar at all sites, but the substrate at low tidal levels was marsh at some sites, and intertidal oyster beds at others. Therefore, the consistent difference among sites was tidal height and not habitat type. The samples were treated as described earlier. The effects of site, tidal level, and sex on reproductive condition were ascertained by ANOVA. Potential differences in GVF among mussels of different sizes were examined using ANCOVA.

The gametogenic condition of mussels from 10 sites in May 1986 is presented in Figure 3. Significant site-to-site variation in reproductive condition was observed ($P < 0.0001$, ANOVA). Despite this variation among sites, high tidal populations had lower GVF values than low tidal populations at all sites. These tidal-related differences in gametogenic condition were highly significant ($P < 0.0001$, ANOVA). A Bonferroni procedure was used to assure 95% confidence in all statements regarding simultaneous pair-wise comparisons of GVF at each tidal level within sites. These comparisons indicate that the differences in GVF of high and low tidal mussels were significant in all but sites 4 (Old Man Creek) and 5 (Town Creek) (Figs. 1, 3). This analysis also suggests that tidal level has a larger effect on reproductive condition than does habitat type. All three habitat types were available at only one site (site 9, Debidue Island). At this site, GVF values of low tidal marsh and low tidal oyster bed mussels were similar, and significantly different ($P < 0.05$) from that of high intertidal mussels (Fig. 3). The evidence for a larger effect of tidal level is further supported by the fact that regardless of habitat type, high tidal mussels had lower GVF values than mussels from the low intertidal at all sites. The observed differences in reproductive activity among tidal levels cannot be explained solely by temperature. Observations at the two primary sites indicate that no major temperature difference occurs between the two tidal levels, and that this difference is not systematic through time (Borrero and Hilbish, unpub. obs.). The temperature variation among sites within the estuary is also very small (LTER Data-base).

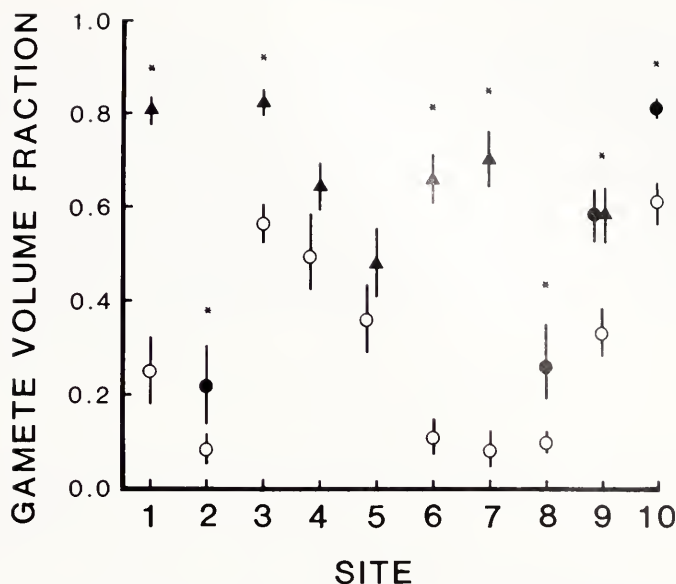


FIGURE 3. Reproductive condition of mussels from high and low tidal populations at ten sites in the North Inlet Estuary, on 24 May 1986. Mean values of total GVF for approximately 15 individuals and the standard error of the mean are plotted for each site and tidal level. Symbols represent mussels from high tidal marsh (○), low tidal marsh (●), and low tidal oyster bed (▲) substrates. The order of sites on the x-axis was established at random. Asterisks indicate significant ($P < 0.05$) differences between tidal levels at each site.

Within the range of sizes considered, ANCOVA did not indicate a significant effect of mussel shell length on reproductive condition, and no difference was found in the reproductive condition of male and female mussels ($P > 0.05$, ANOVA). Examination of the frequency of sexually active (male, female) *versus* undifferentiated mussels indicates again that the onset of gametogenesis is delayed at high tidal locations, compared to populations from the lower intertidal. While all mussels were sexually active at most low tidal populations in May 1986, undifferentiated animals were found at seven of the high tidal populations. The reproductive condition of mussels from the two original populations in the 1983–84 samples was very similar to that obtained from the same populations in 1986.

DISCUSSION

Current understanding of the feeding physiology of suspension-feeding bivalves indicates that the reduction in feeding time experienced by intertidal populations is directly proportional to the duration of aerial exposure. Bivalve molluscs exhibit a limited capacity to compensate for this reduction in feeding time (Bayne *et al.*, in press). The reduction in energy intake by individuals at different tidal levels should be reflected in their productivity patterns. Tidal-related differences in productivity may result in variability of energy allocation to reproduction and timing of reproductive activities.

The results of this study demonstrate that level in the intertidal zone and hence length of submersion and potential feeding time affect the timing of the reproductive cycle of mussel populations. High tidal mussels in the North Inlet Estuary exhibited

delayed gonadal development compared to mussels from the lower intertidal. Spawning was documented only for the two populations from sites 1 and 2, but temporal differences in the onset of gametogenesis and reproductive condition of mussels in May 1983 provide additional evidence that the delay in reproductive activity of high tidal populations is a general phenomenon. Studies on the reproduction of littoral populations of other bivalve species indicate this may be a common pattern. Differences in reproductive maturity of *Cardium edule* from low intertidal and high shore sites were interpreted as due to the difference in synchrony of spawning at varying shore levels (Boyden, 1971). Spawning by intertidal *Modiolus modiolus* occurred between late autumn and winter while it extended through most of the year in a subtidal population from Ireland (Seed and Brown, 1977). Hackney (1983) observed variations in the timing of gonadal activity and spawning between well-flooded and irregularly flooded intertidal populations of *Polymesoda caroliniana* from Mississippi and Florida. Not surprisingly, this pattern seems to apply to other invertebrate groups. Palmer (1980) reported a temporal delay in the reproductive maxima of intertidal *Microarthridion littorale* (Copepoda), compared to a subtidal population at the same site. Similarly, the highest percentage of individuals with egg masses was associated with longer submergence time among two species of barnacles, interpreted as an effect of time available for feeding on reproductive activity (Page, 1984).

A complete comparison of the effects of substrate type upon reproductive condition cannot be achieved with the data from the present study. However, the effect of tidal height was apparent at all sites despite the heterogeneous nature of the substrates. This suggests that tidal height explains a major portion of the overall variance in reproductive condition.

Microgeographic variation in the timing of reproductive activity documented in this study may be as great as that observed among latitudinally separated populations. This is evident from a comparison of the reproductive cycle of populations of *G. demissa* from the east coast of the United States. In Massachusetts, ripe mussels were observed in June–July, and spawning may occur in August–September (Jordan and Valiela, 1982). In Connecticut, gonadal development began in March, fully ripe individuals were observed June through September, and spawning occurred during the summer months (Brousseau, 1982). Spawning took place between August and September in North Carolina (McDougall, 1943), and Kuenzler (1961) indicated that spawning proceeded during July–August and into September in Georgia. No latitudinal pattern of variation appears to exist in the reproductive cycle of *G. demissa*. The results of the present study demonstrate temporal variation in reproductive activity within the North Inlet Estuary, as large as that reported from latitudinally separated localities. Furthermore, temporal variation in reproductive condition among tidal levels at a single site may be larger than variation between localities at greatly different latitudes.

Similar results were obtained by Newell *et al.* (1982) in a study on reproductive variation of *Mytilus edulis*. A latitudinal pattern in the timing of reproductive activity of this species could not be established along the eastern coast of the United States. Site-to-site differences in food quantity and/or quality, and not temperature were identified as the major determinants of the timing of gametogenesis and spawning of *Mytilus* populations (Newell *et al.*, 1982). The present study supports these conclusions. The two mussel populations described here differed two-fold in the time available for feeding, and a similar difference in length of submersion applied for the high and low tidal populations at all sites sampled.

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DIFFERENCES IN THE DURATION OF EGG DIAPAUSE OF *LABIDOCERA AESTIVA* (COPEPODA: CALANOIDA) FROM THE WOODS HOLE, MASSACHUSETTS, REGION

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ABSTRACT

The duration of diapause of *Labidocera aestiva* eggs collected from the field and reared in the laboratory was determined at 5°C. A clear seasonal trend was observed. Diapause eggs produced in the early fall required a much longer exposure to cold to yield a 50% hatch (CT₅₀) (*i.e.*, the duration of diapause was longer) than eggs produced later in the fall. Eggs produced by laboratory animals that were reared at 14°C, 8L–16D, required a shorter period of chilling to terminate diapause than the eggs of animals reared at 19°C, 12L–12D. Considerable variation in the CT₅₀ value was also observed among laboratory cultures that were all reared under identical conditions, but which differed in terms of selection history. The results indicate that both the genotype of the egg and the conditions prevailing during oocyte formation influence the duration of diapause. Eggs that were stored at 5°C for periods longer than 300 days no longer hatched upon warming. It is suggested that the variation in the duration of diapause is an adaptation that promotes synchronization of hatching by ensuring that all individuals terminate diapause at approximately the same time, and survival during the winter by conferring cold-hardiness. Synchronizing the onset of post-diapause development is also discussed as an alternative mechanism for achieving synchronous hatching.

INTRODUCTION

The calanoid copepod, *Labidocera aestiva*, is a seasonal member (summer and fall) of the planktonic community in the Woods Hole region. In this area most *L. aestiva* females have the genetic potential to produce two types of eggs: subitaneous and diapause (Marcus, 1982). Subitaneous eggs are produced during the summer and fall; diapause eggs are produced during the fall. Both egg types begin to develop following their release by females. Subitaneous eggs typically hatch within 1 to 4 days at 21 to 23°C (Marcus, 1979). Diapause eggs enter a refractory phase after 24 to 48 h of development. During the refractory phase, further embryogenesis is not apparent (Marcus, pers. obs.) and diapause eggs cannot be induced to hatch even if conditions are favorable. The duration of diapause (*i.e.*, the length of the refractory period) is positively related to the temperature at which eggs are held (Grice and Gibson, 1975; Marcus, 1979). Once the refractory phase is completed, post-diapause development and hatching occurs if conditions (*e.g.*, temperature) are favorable. For instance, at 21 to 23°C hatching typically occurs within 1 to 2 days (Marcus, 1979). Several field and laboratory studies (Marcus, 1979, 1980, 1984) support the claim that the perpet-

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uation of *L. aestiva* year after year in the Woods Hole region is due to the diapause eggs which overwinter on the sea-bottom and hatch in the spring.

During field and laboratory studies on *L. aestiva* I observed that many diapause eggs could be induced to hatch at 19°C following a chilling period of 4 weeks at 5°C. However, some eggs would hatch with a shorter period of chilling while others required an even longer exposure to cold. A comparison of diapause eggs obtained from females collected in the field showed that the period of chilling that would result in a 50% hatch at 19°C was longer for the diapause eggs of females collected early in the fall (Marcus, 1986). This study examines in more detail the seasonal variation in the duration of diapause of eggs of freshly caught animals from the field and compares the results to values obtained for diapause eggs of females reared in the laboratory. The results indicate that the genotype of an egg and the environmental factors acting during oocyte formation influence the duration of diapause. Based on the results, I suggest that the variation in the duration of diapause is an adaptation that promotes synchronization of hatching in the field by ensuring that all individuals terminate diapause at approximately the same time, and survival during the winter by conferring cold-hardiness.

MATERIALS AND METHODS

Diapause eggs were obtained from animals collected at one to two week intervals over a period of 2 years from October 1981 to October 1983. For each sampling date, adult females were collected from Vineyard Sound by towing a $\frac{3}{4}$ m diameter, 243 μ mesh plankton net for 10 min. Water temperature was determined on a surficial bucket sample for all but two collection dates. For these dates, water temperature was estimated based on the daily temperature record for water off the Woods Hole Oceanographic Institution dock. A comparison of several dates showed that the WHOI values were typically about 1°C less than Vineyard Sound values. Field sampling dates and surface water temperature at the time of collection are shown in Table 1. In the laboratory females were transferred to 100 ml dishes containing 5 μ m-filtered seawater and the dinoflagellate *Gymnodinium nelsoni* (500 cells/ml). The dishes were incubated overnight at 19°C. The next day eggs were collected by pipette, pooled in a separate dish of filtered seawater, and returned to the incubator for 2–4 days to allow the subitaneous eggs to hatch. Unhatched eggs that appeared to be diapause eggs (*i.e.*, the interiors were green, with a clear perimeter) were distributed into 75 ml glass screw capped jars (20–25 eggs/jar) containing filtered seawater, and refrigerated at 5°C. Eggs that were obviously non-viable (*i.e.*, the interiors were brownish, granular, and disintegrating) were discarded. Every 2 to 4 days, a jar was removed (except for the 2 collections in 1981 for which duplicate jars were removed), warmed to 19°C, and held at that temperature. After 4 to 5 days the proportion of hatched eggs was ascertained. The average hatch of the duplicates was recorded for the two 1981 collection dates.

Eggs of laboratory-reared animals were from 12 different cultures that were reared either at a temperature of 14 or 19°C ($\pm 1^\circ$ C), and a photoperiodic regimen of 8L–16D or 12L–12D. The eggs collected from each culture were 1–2 days old. The adults were approximately 2 weeks past reproductive maturity. The cultures represented specific generations of three inbred lines that were being perpetuated as part of a long-term selection experiment designed to assess the potential for evolutionary change in the diapause response threshold (*i.e.*, the necessary conditions for the expression of diapause). Each line was initiated from 500–1000 nauplii that were derived from pooled batches of either subitaneous or diapause eggs produced by 60 females col-

TABLE I

Collection dates, surface water temperature (°C), chilling time (days) required for initial hatch, CT_{50} values (days), and regression parameters pertaining to diapause eggs of field collected females

Date	°C	Initial	CT_{50}	r^2	Slope
9/20/82	19	14	23.19	.88	7.31
9/26/83	21	18	28.82	.80	9.07
9/27/82	19	12	28.61	.79	4.14
10/04/82	18	14	27.40	.88	6.03
10/11/83	18	23	26.13	.90	13.69
10/12/82	17	16	23.02	.78	10.11
10/19/81	14	4	15.44	.89	3.66
10/21/82	14	6	24.14	.74	3.71
10/24/83	16	20	22.37	.84	8.61
11/01/82	14	8	17.30	.91	5.25
11/10/82	13	6	16.53	.90	4.81
11/23/82	11	4	15.36	.80	3.22
11/29/82	10	2	8.98	.89	3.44
12/01/81	7	6	10.99	.99	3.16
12/08/82	10	2	10.17	.96	2.71

lected from the field. Two of the lines were termed subitaneous. Each generation was perpetuated from 500–1000 nauplii derived from just the subitaneous eggs that were produced by the preceding generation of animals. A third diapause line was perpetuated in a similar manner, but from just diapause eggs. The specific rearing conditions and selection histories are shown in Table II. The proportion of subitaneous and diapause eggs produced by each generation of animals varied within and between the lines. The diet for all cultures consisted of a standard mix of four dinoflagellates. General methods for rearing of *L. aestiva* have been described previously (Marcus, 1980). Eggs from each culture were incubated at 19°C for 4 to 5 days after which the diapause eggs were distributed (20 to 30 eggs/jar) into 75 ml jars. The jars were refrigerated at 5°C. At 2 to 4 day intervals the jars were removed, warmed to 19°C, and held at that temperature. The proportion of eggs that hatched after 4 to 5 days was determined.

For each field sampling date and laboratory culture, values of percent hatch were transformed to probit values (Finney, 1952). A regression analysis was performed with these values *versus* the number of days chilled ($\log_{10}$) to derive an estimate of the days of chilling required to promote a 50% hatch (CT_{50}). Calculations were done with an IBM PC and the statistical software package, STATPRO.

The effect of long-term storage at 5°C on egg viability and hatching was examined for the 2 sets of diapause eggs obtained from culture 339. After the initial analysis period, jars of eggs were removed at intervals of up to 4 weeks for more than a year. The hatch of these eggs after warming to 19°C was ascertained as described above.

RESULTS

In general, a shorter period of chilling was necessary to promote initial and 50% hatching of eggs produced by females collected from the field later in the fall (Table I, Fig. 1). The results of the Probit transformation and regression analysis permit a quantified comparison of these differences and the derivation of the median effective chilling period (*i.e.*, the number of days of chilling that promote a 50% hatch). The

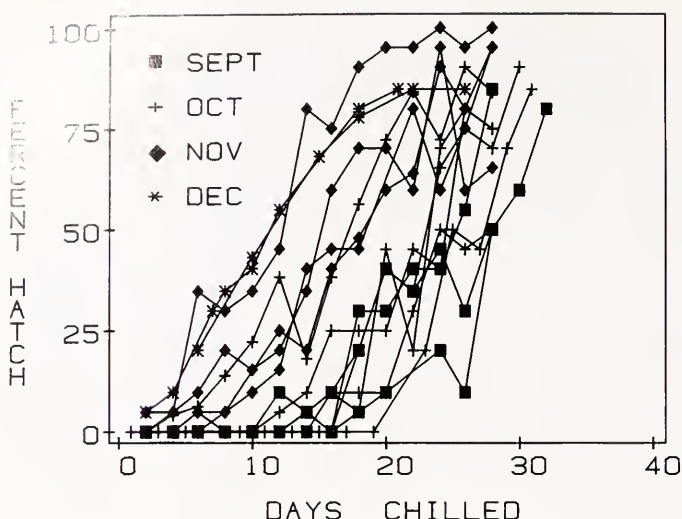


FIGURE 1. Percent hatch of diapause eggs, from field-collected females, at 19°C after chilling at 5°C for the designated number of days. Each set of connected points represents a specific sampling date. Dates for each month are grouped by the indicated symbols.

regression parameters (slope, r^2) and CT_{50} values are shown in Table I. The coefficient of determination values (r^2) ranged from .74 to .99 indicating that the linear regression relationship was a good one for estimating the CT_{50} . For the diapause eggs of field-collected females, the CT_{50} values ranged from 8.98 to 28.82 days. The slope values of the regression ranged from 2.71 to 13.69 probit value/days ($\log_{10}$). This latter parameter provides an indication of the time spread of diapause duration around the median. A high value corresponds to a very short interval for the time from initial to maximal hatching. The highest values tended to occur during September and October, and the lowest during November and December. This same pattern was found for the CT_{50} values. Further analysis revealed that a very good positive correlation ($r^2 = .84$) existed between CT_{50} values and surface water temperature at the time of sampling (Fig. 2).

The median effective duration of chilling also differed among the laboratory reared groups although the range of values was not as great as observed for the field group. The regression parameters (slope, r^2) and CT_{50} values are shown in Table II. As for the field group the r^2 values were high (.71 to .94). The CT_{50} values ranged from 5.82 to 21.09 days. The slope values of the regression ranged from 1.70 to 5.23 probit value/days ($\log_{10}$). The lowest CT_{50} values were obtained for the 3 cultures (370, 371, 374) that were reared at 14°C and 8L-16D. For 2 pairs of cultures, 370 and 372, and 371 and 373, the within pair cultures were established from the same pool of eggs in the 23rd generation, but were reared at the two alternative sets of conditions. In both cases the CT_{50} values were lower for the cultures reared at 14°C, and 8L-16D. A third unpaired culture (374) was reared at 14°C and 8L-16D and also yielded the third lowest CT_{50} value.

The two sets of eggs that were obtained from culture 339 were collected on different days and the CT_{50} values differed by almost 5 days. The long term response to chilling was also different for the two sets (Fig. 3). The hatch after chilling increased more rapidly during the first 30 days for 339b, but a high hatch was maintained for

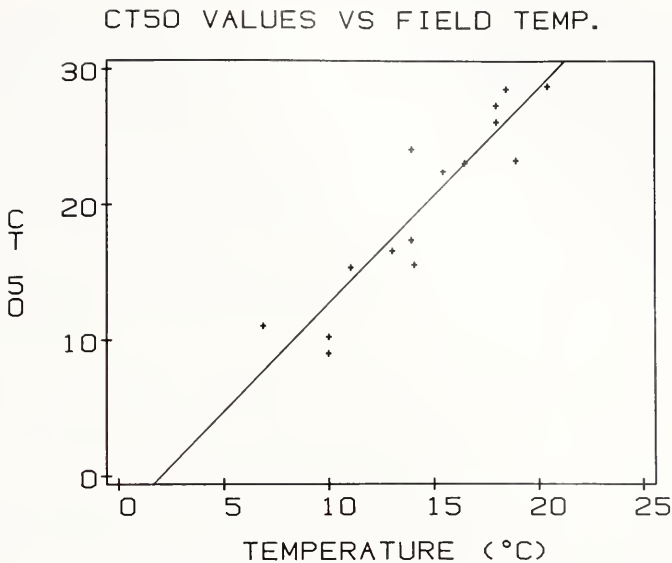


FIGURE 2. Linear regression analysis of CT₅₀ value of each field sample and the surface water temperature at the time of collection.

only 150 to 200 days whereas a high hatch was maintained by the eggs of 339a for almost 300 days. Although the hatch of both sets dropped off to near 0% levels after 300 days, many of the eggs in both sets still appeared viable.

DISCUSSION

This study shows that the median effective number of days of chilling at 5°C decreased as the fall season progressed for the diapause eggs of field-collected females.

TABLE II

Culture # (generation, subitaneous-s or diapause-d line), rearing conditions (photoperiod and temperature), CT₅₀ values (days), and regression parameters pertaining to diapause eggs of laboratory-reared females

Culture #	Conditions	CT ₅₀	r ²	Slope
370 (23s)	14°C, 8L-16D	10.43	.85	2.18
371 (23s)	14°C, 8L-16D	5.82	.71	1.70
374 (6s)	14°C, 8L-16D	10.74	.90	2.92
375 (3s)	19°C, 12L-12D	12.16	.91	2.59
372 (23s)	19°C, 12L-12D	11.77	.78	3.11
373 (23s)	19°C, 12L-12D	14.33	.74	2.37
376 (7d)	19°C, 12L-12D	19.33	.78	2.41
323 (7s)	19°C, 12L-12D	17.92	.83	4.42
329 (15s)	19°C, 12L-12D	13.64	.87	3.04
325 (4d)	19°C, 12L-12D	21.09	.78	3.27
339a (5d)*	19°C, 12L-12D	16.59	.93	5.23
339b (5d)*	19°C, 12L-12D	11.81	.94	3.73
330 (15s)	19°C, 8L-16D	15.66	.84	3.54

* Eggs collected from same culture, but on two different days.

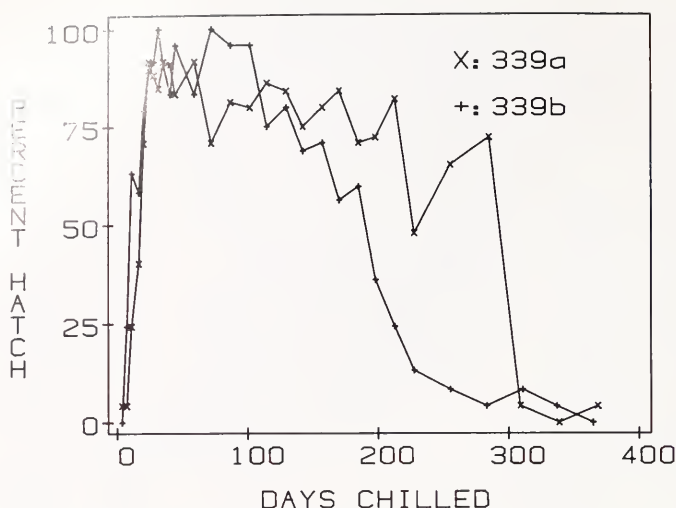


FIGURE 3. Percent hatch of diapause eggs, produced by culture 339 on different days (a and b), at 19°C after chilling at 5°C for the designated number of days.

The range of values spanned 20 days. Similar seasonal trends have been reported for the diapause stages of insects (Burdick, 1937; Church and Salt, 1952). During the collecting period of *L. aestiva*, water temperatures ranged from 20.5 to 6.9°C, a difference of approximately 14.0°C. The two temperatures at which the laboratory animals were reared differed by 5°C and the number of days of chilling required to achieve a 50% hatch differed by as much as 15.5 days. At a constant 19°C, the range in CT_{50} values was about 10 days for the eggs of laboratory-reared animals. Thus for the laboratory-reared animals considerable variation in the median effective days of chilling was obtained, despite the fact that the environmental conditions were the same. This variation must reflect genetic differences. Further evidence for genetic variation are the different responses observed for the eggs of culture 339 that were collected on different days. Since the eggs all came from the same culture, the only possible explanation is that the eggs collected on the different days were produced by different mixes of parents. Although the cultures of animals that were used for the analyses represented different generations of three genetically distinct lines, no obvious association was observed between diapause duration and generation number or selection history.

Although genetic differences appear to be important, environmental factors may also have an effect. The three shortest times to achieve a 50% hatch were obtained for the cultures that had been reared at 14°C and 8L–16D (Table II). This same relationship with temperature and short-daylengths was evident for the eggs of the field collected animals. The work of Denlinger and Bradfield (1981) on the tobacco hornworm provides a possible explanation for these trends. They showed that the duration of diapause was influenced by the number of short day cycles perceived by individuals. As the number of short day cycles experienced by an individual increased, the duration of diapause decreased. They concluded that in the field the duration of diapause is shorter for individuals entering diapause late in the fall because declining temperatures lead to slower development and therefore a longer exposure to short daylengths. If this mechanism characterizes *L. aestiva* it is unlikely that the oocyte

itself could perceive the number of short day cycles. Hence, the effect would have to be mediated through the parent female as a "maternal effect." In the case of *L. aestiva*, it is also possible that declining temperatures directly influence the physiological state of the female and, in turn, oogenesis. The differences observed for the eggs of field-collected animals could also result from variation in maternal age. Animals collected late in the fall might be older than ones collected early in the fall. However, this would not explain the variation expressed by the eggs of laboratory-reared females since all of the animals were similar in age. Krysan and Branson (1977) conducted specific crosses with the corn rootworm and showed that the duration of diapause was affected both by the genotype of the embryo and a maternal component. Further experiments are needed to assess the relative importance of these components in *L. aestiva*.

The long term response to chilling observed for the eggs of culture 339 (Fig. 3) is very similar to patterns observed for several insect species (Hussey, 1955; Cranham, 1972; Lees, 1955). For these species, the percent of individuals terminating diapause increased to a maximum with increasing length of exposure to cold, then remained high with longer exposure to cold, and finally declined with excessive time of exposure to cold. In each case the percent terminating diapause remained low after excessive exposure, but the interpretation of the results differed among authors. Hussey (1955) suggested that the decline was part of an annual cycle and that given enough time the percent terminating diapause would increase again. Hussey believed that there was an internal gating rhythm that controlled emergence from diapause. However, since he did not carry the experiments through for another year it cannot be certain that death had not occurred. The rhythm concept was not discussed by the others despite similar results. They concluded that the eggs had lost viability and would never hatch. It would certainly be advantageous for an egg of *L. aestiva* to remain viable beyond one season. *L. aestiva* eggs that are buried do not hatch despite favorable temperatures (Marcus and Schmidt-Gengenbach, 1986). The probability of completing development should be higher for a diapause egg (from the previous fall) that is uncovered no later than the summer than for an egg which is not uncovered until October or November. If hatching occurred only in October and November, the likelihood of completing development should be diminished due to declining temperatures. A gating rhythm that controlled the onset of post-diapause development would reduce the probability of eggs hatching at an inappropriate time. However, this study does not support such an hypothesis. After 300 days of chilling, the percent hatch of *L. aestiva* eggs after warming declined. After more than 400 days of chilling, hatching has not increased again though many of the eggs look viable. Thus this study indicates that eggs cannot survive more than 300 days of constant exposure to 5°C in the laboratory. However, this life span may be quite different in the field, where eggs probably experience long periods of anoxia and exposure to hydrogen sulfide. Although the effect of such parameters on the viability of *L. aestiva* eggs is not known, it has been reported (Uye *et al.*, 1984) that exposure to organic pollution reduces the viability of resting eggs of neritic marine copepods.

From studies of insects and freshwater copepods, I suggested (Marcus, 1979) that *L. aestiva* eggs terminate diapause at different times during the winter in the field, and are held at a stage of pre-hatch readiness because water temperatures are below the threshold for post-diapause development and hatching. This study supports that hypothesis, although the duration of diapause under field conditions appears to differ from that observed in the laboratory where temperature was held constant at 5°C. The data (Marcus, 1984) for eggs collected from bottom sediments in Buzzards Bay, Massachusetts, indicated that the refractory phase was not completed by all eggs in

December. It was suggested that the eggs which failed to hatch at this time were produced later in the fall and had not completed the refractory period. By February all eggs appeared to have completed the refractory phase as evidenced by the high hatch of eggs incubated at 19°C. The present study does not support that suggestion. Because of the seasonal variation in the duration of diapause it is possible that eggs which were collected from sediments in December and did not hatch in the laboratory upon warming, were produced in September as well as in December.

Temperature is not the only environmental parameter that affects the transition from diapause to development. Hatching is also affected by light and oxygen concentration (reviewed by Grice and Marcus, 1981). Although the effect of these parameters on the termination of diapause and the onset of post-diapause development in marine copepods has not been clarified, Brewer (1964) reported that exposure to reduced oxygen concentrations was necessary to terminate egg diapause in the freshwater copepod, *Diaptomus stagnalis*, and Watson and Smallman (1971) suggested that photoperiod was a necessary cue for the resumption of development in *Diacyclops navus*. The transition from dormancy to development is mediated by pH in brine shrimp (Busa and Crowe, 1983).

Diapause is an important factor in the synchronization of life cycles (Tauber *et al.*, 1986). Two possible ways in which synchronization can be achieved are synchronizing the termination of diapause and synchronizing the onset of post-diapause development and hatching. Both mechanisms characterize *L. aestiva* in Woods Hole waters. Since diapause eggs are produced over a span of several months, the longer diapause of eggs produced early in the fall ensures that they do not terminate diapause until winter temperatures have declined below the threshold for post-diapause development. Conversely, the shorter diapause of eggs produced late in the fall ensures that diapause will be completed prior to the time when water temperature exceeds the threshold in the spring. Since diapause in the field terminates by February or March in Woods Hole waters (Marcus, 1984) and hatching does not occur until May (Grice and Gibson, 1975) the precise coincidence of diapause termination should be less important than the coincidence of the onset of post-diapause development in the promotion of synchronous hatching. Thus, as long as the refractory phase is completed before the threshold for post-diapause development or hatching is exceeded, synchronization of hatching should still occur. The coincidence of diapause termination among overwintering eggs may be more critical at more southern latitudes where water temperatures at the time of diapause termination are more likely to exceed the threshold and thus individuals would resume development as soon as the refractory period ended. The results also suggest that diapause is important because it promotes the survival of individuals by conferring cold-hardiness. Diapause eggs which complete their refractory period by January (Marcus, 1984), can tolerate exposure to cold winter temperatures. However, subitaneous eggs do not survive extended exposure to such temperatures (Grice, unpub.). I suggest that the variation in diapause duration expressed by *L. aestiva* is an adaptation that promotes synchronization by ensuring that all individuals terminate diapause at approximately the same time, and survival by conferring cold-hardiness.

ACKNOWLEDGMENTS

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CYTOCHEMICAL FEATURES OF SHRIMP HEMOCYTES

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ABSTRACT

Morphological studies suggest that there are several types of decapod hemocytes; however, distinguishing criteria based on conventional staining techniques are often subtle or ambiguous. Cytochemical features of ridgeback prawn (Penaeidae: *Sicyonia ingentis*) hemocytes were studied using specific stains for lysosomes, cytoplasmic contents, and granule enzymes. This approach facilitates the differentiation of cell types in the ridgeback prawn and provides information on the functions of and relationships among different cell types.

Agranular hemocytes and a subgroup of small granule hemocytes contain extensive cytoplasmic glycoprotein deposits which display smudgy, intense staining with Sudan black B. As previously shown, coagulogen—the clotting material in decapods—stains with Sudan black B when extracted from lysed hemocytes. Other hemocyte types display light staining limited to granule membranes.

Lysosomes are not observed in agranular cells and are rarely present in small granule hemocytes with glycoprotein deposits. Small granule hemocytes without deposits and large granule hemocytes contain numerous lysosomes as shown by the presence of acid phosphatase, β -glucuronidase, and nonspecific esterase. Acid phosphatase is observed in the Golgi body of these cells, within small vesicles, and in small granules. The granules in large granule hemocytes rarely show acid phosphatase reaction, yet small acid phosphatase-positive vesicles fuse with the large granules. The acid phosphatase in the large granules may exist in an inactive form. Prophenoloxidase activity is localized only in large granules. The physiological significance of hemocyte cytochemistry is also discussed.

INTRODUCTION

Crustacean hemocytes perform a variety of physiological and pathological functions including coagulation (Ravindranath, 1980), phagocytosis, recognition of foreign material, carbohydrate transport, and encapsulation (Bauchau, 1981). Several hemocyte categories have been recognized in decapod crustaceans based on morphological criteria. Morphological features are often subtle and ambiguous, and are not readily recognized by other investigators. In addition, morphological criteria are rarely based on properties that facilitate the differentiation between stages in hemocyte maturation or among cells with different physiological functions. Hence, previous investigations have failed to define a clear correspondence between various cell types and their functions.

In an attempt to develop a comprehensive description of crustacean hemocyte formation and function, cytochemical techniques were used to complement our previous morphological description of shrimp hematopoietic tissue (Martin *et al.*, 1987) and circulating hemocytes (Martin and Graves, 1985). Electron microscopic exami-

nation of hemocytes from the ridgeback prawn (Penaeidae: *Sicyonia ingentis*) shows the existence of four cell types: agranular, small granule with cytoplasmic deposits, small granule without cytoplasmic deposits, and large granule hemocytes. Agranular hemocytes are small cells with a high nucleus:cytoplasm ratio (Martin *et al.*, 1987). Their cytoplasm contains little other than aggregations of electron-dense deposits. A subset of small granule hemocytes contains similar electron-dense cytoplasmic deposits, one to six round striated granules, and occasional electron-dense granules. In contrast, a distinct subset of small granule hemocytes and the large granule hemocytes lack cytoplasmic deposits and striated granules. These hemocytes have many (>10) electron-dense, electron-lucent, or punctate granules which range in diameter from $0.4\ \mu\text{m}$ in small granule hemocytes to $0.8\ \mu\text{m}$ in large granule cells. Intermediate stages were observed between agranular hemocytes and small granule hemocytes with deposits and between small granule hemocytes without deposits and large granule hemocytes, suggesting the existence of two distinct hemocyte lines.

In view of the difficulty in accurately identifying certain hemocyte categories at the light microscope level, various enzymatic and cytochemical methods were evaluated for use in hemocyte classification. The goals of this study are to (1) identify cytochemical stains which can be used to differentiate specific hemocyte types, and (2) provide useful information on the function of the various cell types.

MATERIALS AND METHODS

Animals

Ridgeback prawns were collected and maintained as previously described (Martin *et al.*, 1987). Shrimp averaged 14.5 g and were in molt stages C and D (Anderson, 1985).

Tissue collection and preparation

Hemolymph (usually 0.2 cc) was withdrawn from the ventral sinus or heart into a 1 cc syringe containing anticoagulant (Martin and Graves, 1985). Hemocyte smears were then prepared on glass microscope slides, allowed to air dry, and used for light microscopy.

Hemocytes and hematopoietic nodules to be examined at the electron microscopic (EM) level were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.8) containing 12% glucose for 1 h at room temperature. Following a 30 min wash in 0.1 M sodium cacodylate (pH 7.8) containing 24% sucrose, the tissues were post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate for 1 h at room temperature, dehydrated in a graded series of ethanol, and infiltrated and embedded in Spurr's (1969) low viscosity plastic.

Epigastric hematopoietic nodules were dissected from shrimp as described by Martin *et al.* (1987). Touch preparations of sagittally cut nodules were air-dried prior to the cytochemical demonstration of prophenoloxidase (Ppo). Frozen sections ($7\ \mu\text{m}$ thick) were cut using a Tissue Tek II cryostat for the demonstration of lysosomal enzymes. Thin sections ($7\ \mu\text{m}$) were also prepared using formalin-fixed, paraffin-embedded tissue.

Demonstration of cytoplasmic constituents

Following a two-minute fixation in absolute ethanol, smears were stained with bromphenol blue or periodic acid-Schiff (PAS). Smears to be stained with Best's car-

mine (Sheehan and Hrapchak, 1980) were fixed in ethanol for 30 minutes. PAS and carmine were compared with and without prior digestion by α -amylase. For the digestions, hemocytes were suspended in 0.5% aqueous amylase for 1 h, then pelleted by a 5 min centrifugation at $500 \times g$ in a table top centrifuge before preparation of the smear. Sections of hematopoietic nodule were stained with bromphenol blue or PAS.

Enzymatic extractions to demonstrate composition of cytoplasmic deposits and granules were also examined using EM. Hemocytes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.8) containing 12% glucose for 1 h, then washed in cacodylate buffer and kept at 4°C for 12 to 18 h. The hemocyte pellet was then dehydrated through a graded ethanol series, infiltrated and embedded in Spurr's (1969) low viscosity plastic. Thin sections were cut on a Porter Blum MT2B ultramicrotome, picked up on gold grids, and floated on one of the following solutions for 2 to 20 h at 37°C: (A) 0.5% protease in 0.2 M phosphate buffer (pH 7.4) or (B) 0.5% α -amylase in 0.2 M phosphate buffer (pH 7.4). These sections and control sections (floated on distilled water for an equivalent period of time) were examined unstained and stained (0.5% uranyl acetate in 0.05 M Tris-maleate for 1 h at room temperature) using a Hitachi HU11A transmission electron microscope.

Lipids were demonstrated in hemocyte smears and nodule touch preparations using a commercial Sudan black B kit (Sigma Chemical Co. Kit #380) according to provided directions.

Prophenoloxidase activity

To test for the presence of prophenoloxidase (Ppo), hemocytes and hematopoietic tissue touch preparations were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C. The cells were given three 15-min rinses in phosphate buffer, incubated in 0.1% L-DOPA in phosphate buffer for 16 h at room temperature (Soderhall and Smith, 1976), and examined by light microscopy.

Lysosomal enzymes

The presence of acid phosphatase (Sigma Chemical Co. Kit #386), β -glucuronidase (Kit #180), and α -aryl naphthyl esterase—a nonspecific esterase (Kit #90)—were demonstrated at the light microscopic level using commercial research kits (Sigma Chemical Co.). Hemocyte smears and frozen sections of hematopoietic nodule were fixed in glutaraldehyde and incubated according to provided directions. Staining patterns for each enzyme were quantified at $1000\times$ by estimating the numbers and sizes of positive areas in 10 cells from each of the 4 hemocyte categories described by Martin *et al.* (1987).

To localize acid phosphatase at the EM level, fixed hemocytes and hematopoietic nodules were washed thoroughly and then incubated in a medium consisting of 40 mM Tris-maleate buffer (pH 5), 11.5 mM sodium β -glycerophosphate, 2.4 mM lead nitrate, and 5% sucrose at 37°C for 2 h. Hemocytes were then processed as described above.

The same procedure was followed for glucose-6-phosphatase, alkaline phosphatase, and peroxidase except for the use of different incubation media. For glucose-6-phosphatase, the fixed cells were incubated in a medium composed of 25 mg glucose-6-phosphate, 27 ml distilled water, and 20 ml of 0.3 M Tris-maleate buffer (pH 9.7). The incubation medium for alkaline phosphatase consisted of 4 ml 1.25% sodium β -glycerophosphate, 4 ml of 0.2 M Tris-maleate buffer (pH 9), 9.4 ml distilled water, and 2.6 ml of 1% lead nitrate. The peroxidase medium contained 5 mg 3,3-diamino-

benzidine tetrahydrochloride, 10 ml of Tris-maleate buffer (pH 7.6), and 0.1 ml of 1% H_2O_2 .

RESULTS

Cytoplasmic constituents

The abundant cytoplasmic deposits of agranular hemocytes and a subset of small granule hemocytes are composed of glycoproteins as evidenced by positive reactions with PAS, carmine, and bromphenol blue. Digestion with α -amylase prior to application of PAS and carmine reduced but did not completely remove the staining of these cytoplasmic deposits. Tissue sections of the epigastric hematopoietic nodule stained with PAS or bromphenol blue yields results similar to those in free hemocytes.

Sudan black B produces a distinctive staining pattern in agranular hemocytes and small granule hemocytes with cytoplasmic deposits (Figs. 1, 2). These cells appear smudgy, with the heavy dark stain obscuring nuclear characteristics. Only a thin clear zone adjacent to the plasma membrane is occasionally present. Staining of the cytoplasmic deposits by Sudan black B indicates the presence of a lipid moiety associated with the glycoprotein. In small granule hemocytes lacking deposits (Fig. 3) and in large granule hemocytes (Fig. 4), delicate staining is evident only around granule and nuclear membranes, producing a diffuse pattern. Maturing hemocytes from the hematopoietic nodule display identical staining patterns.

Granule histochemistry

Granules in free and maturing hemocytes are stained with PAS, carmine, and bromphenol blue, indicating the presence of glycoproteins. Prior amylase digestion removes granular staining by PAS and carmine.

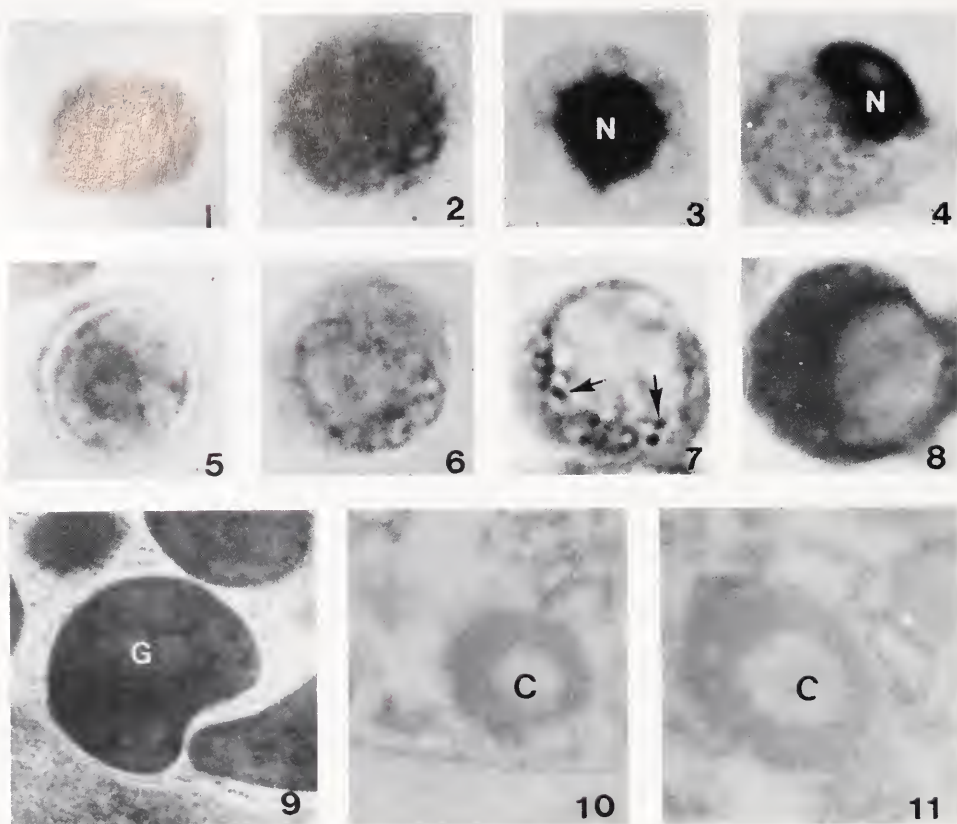
Prophenoloxidase activity is visualized following incubations of fixed hemocytes and hematopoietic tissue touch preparations with L-DOPA (Figs. 5–8). Ppo activity is limited to granules of small granule hemocytes lacking glycoprotein deposits and large granule hemocytes. In some animals (molt stage D), almost 100% of these cell types display intense activity (>10 positive granules each) while in intermolt shrimp, less than 1% of these cells are positive. Similar results are obtained using hemocyte smears and tissue touch preparations.

No peroxidase activity is observed in any of the hemocyte categories.

The glycoprotein content of large and small granules is also seen in sectioned tissues that were subsequently treated with protease or α -amylase. Figure 9 shows a large granule from a hemocyte viewed after standard preparation. Figures 10 and 11 show granules in sections treated with α -amylase (12 h) and protease (6 h). At these times, the core of the granules has been extracted, however, with longer incubations (20 h), the entire granule is extracted by both enzymes.

Lysosomal enzymes

Three hydrolases (acid phosphatase, β -glucuronidase, and nonspecific esterase) were used to demonstrate the presence of lysosomes in hemocytes at the light microscope level (Table I). These stains yield similar cytochemical information for each specific hemocyte type although individual hydrolases produce slightly different staining patterns. Agranular hemocytes do not contain any of the lysosomal enzymes. Glycoprotein-rich small granule hemocytes exhibit between zero and three focally positive areas consistent with the size of lysosomes. These cells occasionally contain



FIGURES 1-4. Light micrographs of agranular, small granule hemocyte with deposits, small granule hemocyte without deposits, and large granule hemocyte, respectively, treated to show sites of prophenoloxidase. The first two cells have no reaction product. The granules (arrows) in the small granule hemocyte without deposits react as does the entire cytoplasm of the large granule hemocyte. All figures 2500 $\times$.

FIGURES 5-8. Light micrographs of same cell types as in Figures 1-4, treated with Sudan black B. Agranular and small granule hemocytes with deposits show dense reaction products in the cytoplasm which obscure the nucleus. The latter two cell types have minimal staining and the nucleus (N) is clearly observed. All figures 2500 $\times$.

FIGURE 9. Transmission electron micrograph showing homogeneous and electron-dense granules (G) from a large granule hemocyte fixed with both glutaraldehyde and osmium and stained with uranyl acetate and lead citrate. 43,000 $\times$.

FIGURE 10. Transmission electron micrograph showing a granule from a large granule hemocyte that was fixed only with glutaraldehyde. Thin sections were floated on a protease solution for 6 h and examined without stain. Note the low electron density and extraction of the granule core (C). 43,000 $\times$.

FIGURE 11. Transmission electron micrograph of a granule from a large granule hemocyte prepared as in Figure 10 and then floated on a solution of α -amylase for 12 h. Note the low electron density of the granule and extraction of its core (C). 43,000 $\times$.

a few acid phosphatase-positive granules as well. In contrast, small granule hemocytes lacking cytoplasmic deposits have from three to eight positive foci consistent with lysosomes. Half of these cells have only a few (0-3) positive granules while the remaining small hemocytes contain over 30 positive granules. A few of the latter group, presumably transitional to large granule hemocytes, also exhibit a few large acid phosphatase-positive granules. In the large granule hemocytes, up to three focally positive

TABLE I

Distribution of lysosomal enzymes in shrimp hemocytes

Hemocyte type	Acid phosphatase	β -glucuronidase	Glucose-6-phosphatase	Non-specific esterase	Alkaline phosphatase
Agranular	None	None	None	None	None
Small granule hemocyte with deposits	Rare (1-3 RS*/Cell)	Rare (1-3 RS/Cell)	None	Few (1-10 RS/Cell)	None
Small granule hemocyte without deposits	Mixed (50% of cells have > 30 RS/Cell 50% of cells have 1-10 RS/Cell)	Many (> 10 RS/Cell)	None	Intermediate (10-30 RS/Cell)	None
Large granule hemocytes	Mixed (50% of cells have 0-1 RS/Cell and nuclei are pycnotic 50% of cells have 4-8 RS/Cell)	Many (> 10 RS/Cell)	None	Many (> 30 RS/Cell)	None

* RS stands for reaction sites.

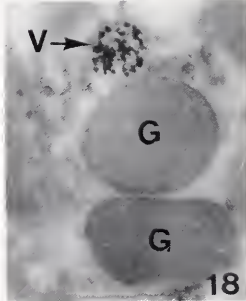
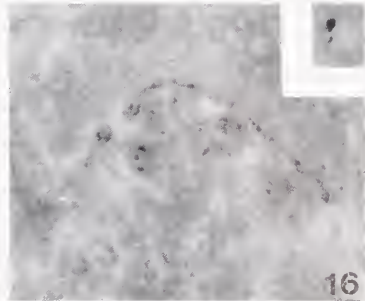
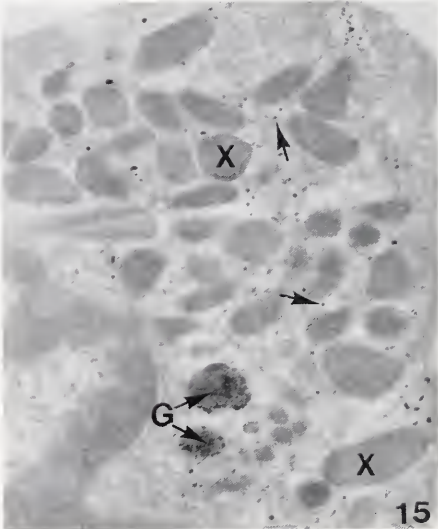
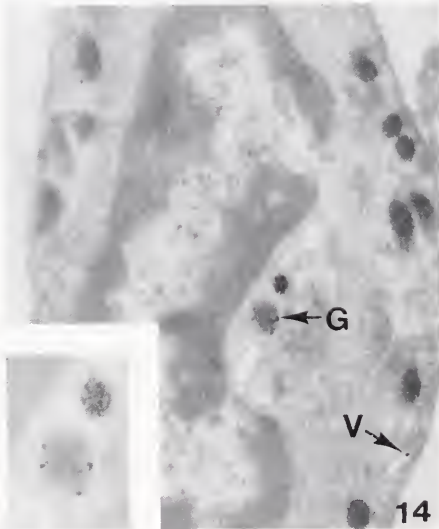
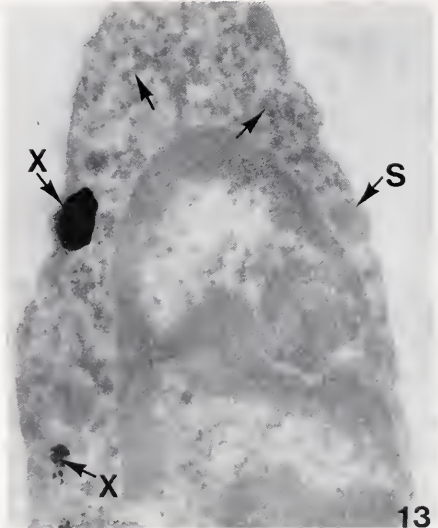
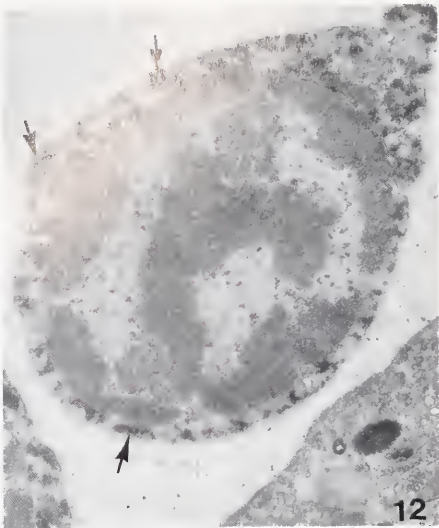
areas consistent with lysosomes were observed. From zero to five small granules are positive as well as from zero to two large granules. Among the large granule hemocytes, the largest cells which contain eccentrically placed, pycnotic nuclei were usually acid phosphatase-negative or contain only one positive focus.

Electron microscopy localization of acid phosphatase yields similar results with no reaction product detected in agranular hemocyte (Fig. 12). Staining is infrequently observed in small granule hemocytes containing glycoprotein deposits and is restricted to small vesicles and granules of the non-striated variety (Fig. 13). Heavy staining is found in the granules of the small granule hemocyte lacking deposits (Fig. 14). Large granule hemocytes have reaction product dispersed throughout the cell in vesicles and the smaller granules. Only a few of the large granules stain positive although these were morphologically indistinguishable from non-reactive granules (Fig. 15). In Figure 16, acid phosphatase-positive trans cisternae and small vesicles are shown budding from a Golgi body. These vesicles (Fig. 16, inset) appear to progressively coalesce, forming larger reaction vesicles (Fig. 17), then small granules, and finally large granules (Fig. 18).

Nonspecific esterase is observed only in granulated cells. Glycoprotein-rich small granule hemocytes are completely negative or contain up to 10 tiny positive areas consistent with the size of vesicles. In contrast, from 10 to over 30 positive vesicles are observed in small granule hemocytes without cytoplasmic deposits. Large granule hemocytes contain numerous (>30) positive vesicles. Patterns of β -glucuronidase staining in granulated cells are similar to those of nonspecific esterase in addition to the presence of a few (<3) positive foci of lysosomal size in the small granule hemocytes.

Maturing hemocytes from frozen sections of the hematopoietic nodule were examined for the presence of acid phosphatase and β -glucuronidase. Staining patterns of acid phosphatase are identical between maturing hemocytes and those described above for free hemocytes. β -glucuronidase activity is not observed in touch preparations of the hematopoietic nodule.

All hemocytes are negative for alkaline phosphatase and glucose-6-phosphatase.



DISCUSSION

Results of cytochemical tests support the morphological classification of ridge-back prawn hemocytes previously developed in our laboratory (Martin *et al.*, 1987) and yield information on the physiological functions performed by the various hemocyte types. A combination of two or three cytochemical tests is suggested for classification of shrimp hemocytes. Sudan black B produces a distinctive smudgy staining pattern in agranular hemocytes and small granule hemocytes with cytoplasmic deposits. Acid phosphatase can be used to differentiate agranular cells, which are negative for lysosomal enzymes. Prophenoloxidase activity is limited to small granule hemocytes without cytoplasmic deposits and large granule hemocytes; however, significant activity may only be demonstrable during the D stage of the molt cycle (Bauchau, 1981).

The glycoprotein deposits in the cytoplasm of agranular hemocytes and a subgroup of small granule hemocytes are distributed in linear arrays throughout the entire cell and are evident in all molt stages (unpub. obs.). In contrast, glycogen—which it resembles ultrastructurally—is typically confined to one area of decapod hemocytes and does not have a linear arrangement (Johnston *et al.*, 1973; Bauchau, 1981). Glycogen has been shown to be transported by hemocytes (Johnston *et al.*, 1973; Bauchau, 1981) and may involve the enzyme glucose-6-phosphatase (Johnston and Davies, 1972). This enzyme, however, was not detected in shrimp hemocytes. The glycoprotein may contain a lipid moiety since the deposits are intensely stained by Sudan black B. Such chemical properties are consistent with those of the primary coagulation protein, coagulogen (Durliat, 1985). In decapod hemocytes, intracellular coagulogen does not appear to be localized in granules although granules are necessary for coagulation to occur (Ravindranath, 1980; Durliat, 1985). Shrimp small granule hemocytes with lipoglycoprotein deposits contain granules with a striated or concentric substructure (Martin *et al.*, 1987). Similar granules have been observed in *Limulus* (Copeland and Levin, 1985), crabs (Bodammer, 1978), lobsters (Hearing and Vernick, 1967; Goldenberg *et al.*, 1986), and crayfish (Unestam and Nylund, 1972), and alterations in the striated granules of shrimp have been observed early in the process of hemolymph coagulation (unpub. obs.).

Lysosomes were observed in each cell type except for agranular hemocytes. Small

FIGURES 12 AND 13. Transmission electron micrographs of an agranular hemocyte (Fig. 12) and a small granule hemocyte with deposits (Fig. 13) treated to display sites of acid phosphatase activity. No reaction sites are present in agranular cells. In small granule hemocytes with deposits, reaction sites (X) are rare and then localized to granules of the electron-dense variety. Striated granules (S) are never labelled. In both cells, note the small amount of cytoplasm which contains the deposits (arrows). Both figures 20,000X.

FIGURE 14. Transmission electron micrograph of a small granule hemocyte that lacks deposits showing a few reaction sites for acid phosphatase in granules (G) and vesicles (V). 20,000X. Inset shows a higher magnification (43,000X) micrograph of the small granules.

FIGURE 15. Transmission electron micrograph of a large granule hemocyte showing reaction sites for acid phosphatase in vesicles throughout the cytoplasm (arrows) and some of the granules (G). Other granules (X) show no reaction product. 20,000X.

FIGURE 16. Transmission electron micrograph showing a Golgi body in a large granule hemocyte. The trans-cisternae contains the acid phosphatase reaction product. 42,000X. Inset shows a vesicle with reaction product. Similar vesicles are commonly seen around Golgi bodies as well as throughout the cytoplasm. 42,000X.

FIGURE 17. Transmission electron micrograph showing acid phosphatase reaction product in three vesicles of increasing diameter. Note how the smallest vesicle appears to be fusing (arrow) with the medium sized vesicle and that the contents of the vesicles are not as electron dense as fully mature granules. 32,000X.

FIGURE 18. Transmission electron micrograph showing acid phosphatase reaction product in one large vesicle (V) and not in two adjacent granules (G). 32,000X.

granule hemocytes with glycoprotein deposits contained only one to three lysosomes per cell. In small granule hemocytes without deposits and in large granule hemocytes, many lysosomes were identified using LM and EM cytochemistry. Although the granules in these cells are morphologically indistinguishable, they may be enzymatically heterogeneous (see Bauchau, 1981). Using TEM, acid phosphatase was localized in some but not all of the granules of the shrimp. The same results were observed in granulocytes of the clam *Mercenaria mercenaria* (Yoshino and Cheng, 1976) and interpreted to indicate a heterogeneous population of granules or a non-synchronized cycle of granule production, perhaps with the final enzyme stored in an inactive form. The presence of numerous lysosomes in the large and small granulocytes which lack deposits supports the suggestion that these cells are phagocytic (Bauchau, 1981; Soderhall *et al.*, 1986) and that the granules are available for intracellular degradation processes. However, because of the large number of granules in a single hemocyte, it is unlikely that granules could be exclusively reserved for phagocytosis. Other researchers suggested extracellular functions for these granules, including recognition of foreign material (Soderhall and Smith, 1983) and agglutinin sequestration (Stang-Voss, 1971).

The recognition of foreign material in arthropods is mediated by the prophenoloxidase system which is located in the granules (Soderhall and Smith, 1983). Results of the present study show that only large granule hemocytes and small granule hemocytes without deposits contain prophenoloxidase activity. Soderhall and Smith (1983) obtained similar conclusions of Ppo activity within granular hemocytes in the crab, *Carcinus maenus*. Ppo activation and exocytosis in response to endotoxin or β -glucan exposure can initiate the coagulation cascade and serves as the crustacean equivalent to the alternate complement and properdin pathways in mammals (Durlat, 1985). The proposed existence of the Ppo system in the small granule hemocytes without deposits—large granule hemocyte line and the coagulation enzymes in the other hemocyte line suggests cooperativity among shrimp hemocytes during endotoxin-mediated coagulation analogous to that observed for defense reactions in insects (Ratcliffe *et al.*, 1984) and crustaceans (Soderhall *et al.*, 1986).

Based upon results of morphological (Martin *et al.*, 1987) and cytochemical studies, shrimp hemocytes can be divided into two cell lines, the deposit line (composed of agranular and striated granule hemocytes) and the granulocyte line (small and large granule hemocytes). Although the lysosomal enzyme data presented here are consistent with maturing stages of a single cell line, the following arguments support our theory: (1) striated granules are never found in granulocytes (The striated granule shown in Fig. 7B of Martin and Graves, 1985, was taken from a small granule hemocyte containing cytoplasmic deposits. However, at that time, no distinction was made between the deposit and granulocyte lines.); (2) glycolipoprotein deposits are never observed in granulocytes; (3) mitosis is observed both in agranular hemocytes and in small granule hemocytes which lack cytoplasmic deposits; (4) cells are present as a continuum of differentiation between agranular and striated granule hemocytes and between small and large granule hemocytes; and (5) clusters of deposit cells and granulocytes are usually segregated within the hematopoietic tissue. The utility of this classification scheme must now be determined by functional studies identifying the role of the various hemocyte types in crucial biological processes such as coagulation, defense reactions, wound healing, and exoskeleton hardening.

ACKNOWLEDGMENTS

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IMPULSE PROPAGATION AND CONTRACTION IN THE TUNIC OF A COMPOUND ASCIDIAN

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ABSTRACT

Diplosoma listerianum and *D. macdonaldi* (Fam. Didemnidae) have a network of cells ("monocytes") in the tunic which contain high concentrations of microfilaments and react positively with NBD-phalloidin, indicating the presence of F-actin. The tunic is contractile, especially in the areas around the cloacal apertures, which can be closed completely. Myocytes are concentrated in sphincter-like bundles around these openings, but also are found throughout the tunic. Electrophysiological recordings reveal a diffuse conduction system in the tunic propagating all-or-none impulses ("tunic potentials," TPs) through all parts with a conduction velocity of $<1.5 \text{ cm} \cdot \text{s}^{-1}$, and a refractory period of 1.6 s. TPs correlate one-for-one with contractions. The system is excitable to the touch, but is also spontaneously active, showing steady patterns of potentials as well as regular, 'parabolic' bursts. The evidence suggests that the myocyte net itself conducts the impulses triggering the contractions. In the absence of conventional nerves and muscles, the system provides the colony with a way of regulating the effluent water current and hence the volume of a common cloacal space.

The TP system is not 'wired in' to the ascidiozooids either as a sensory or as a motor pathway. The tunic acts as an independent behavioral entity.

INTRODUCTION

The ascidian tunic or test is "an outer covering which completely surrounds the individual zooid in solitary ascidians or forms a common groundwork in which the zooids are embedded in colonial species." (Goodbody, 1974). It is a secretion product of the body wall epithelium and consists of a matrix of proteins and carbohydrates (including cellulose) into which cells migrate from the hemocoel during development. Blood vessels often penetrate the tunic, and sensory processes from receptors whose cell bodies lie in the underlying epithelium may also extend into the tunic (references in Bone and Mackie, 1982) but muscles and nerves¹ are absent. The various cells present may be concerned with secretion of tunic materials, phagocytosis, self-nonsel self discrimination, coloration, and some other less well understood functions. Some tunic cells are capable of movement and have contractile pseudopodia or filopodia, but the contractions reported are very slow ($<114 \mu\text{m}$ per hour in *Botryllus* according to Izzard, 1974). Several authors (e.g., Saint-Hilaire, 1931; Godeaux, 1964) have likened the tunic to mesenchyme. Brien (1966) calls it "a living envelope, equivalent to a sort of peripheral mesenchyme." Unlike mesenchyme, however, it is not

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¹ There appears to be only one report of nerve cells in the tunic of an ascidian, that of Das (1936). No later study on tunic histology supports this claim.

covered by epithelium but is exposed to the environment, and in this respect it more resembles a cuticular or exoskeletal tissue.

Given the absence of nerves and muscles from the tunic, it is not surprising that there have been no reports that the structure responds to stimulation, contracts, or 'behaves' in the usual sense, although in several cases it is composed of a fairly plastic, viscous material capable of short-term conformational changes (Della Valle, 1908; Godeaux, 1964). During observations on *Diplosoma listerianum*, however, it became clear that this species has a tunic in which electrical signals propagate on an all-or-nothing basis, mediating contractions of the tunic itself. In this report, the electrophysiological characteristics of this conduction system are described, along with an account of the activities performed and of the cells likely responsible for conduction and contraction. The evidence implicates a novel type of cell ("myocyte") as the basis for both conduction and contraction. These cells seem to combine the properties of conventional nerves and muscles including the ability to function as pacemakers. They are distributed throughout the whole tunic in the form of a dense network which, it is proposed, constitutes the structural basis for the behavioral action system whose electrical correlates are picked up with recording electrodes.

MATERIALS AND METHODS

Two species of *Diplosoma* were used in this study. *D. listerianum* Milne-Edwards, 1841, was obtained at the Stazione Zoologica in Naples, Italy. A species tentatively identified as *D. macdonaldi* Herman, 1886 was obtained at the Friday Harbor Laboratories of the University of Washington, and at the Bamfield Marine Station, Bamfield, British Columbia, Canada. *D. macdonaldi* and *D. listerianum* are very similar and may be conspecific (Monniot, 1974). The specimens collected at Naples grew on the walls of the public display aquarium and elsewhere in the seawater system, where they appear to be endemic. *D. macdonaldi* specimens were collected from rocks and pilings in the intertidal zone. Following the method of Della Valle (1908), specimens were removed from their natural substrates and transferred to glass slides or petri dishes. There they attached after a few hours, subsequently resumed growth, expanded and put out new attachment structures ("crampons"). All the experiments reported in this paper were performed on transplanted specimens maintained in running seawater in the laboratory. The bulk of the work was done at Naples, and *D. listerianum* was used for all the illustrations except Figures 2, 8, and 9.

For histological study, pieces of tunic were dissected out and mounted as whole mounts either fresh or after fixation and examined by phase contrast or Nomarski differential interference contrast microscopy. NBD-phalloidin (from Molecular Probes Inc., 24750 Lawrence Road, Junction City, Oregon 97448) was used as a specific fluorescent stain for F-actin. Material was embedded in Epon 812 for electron microscopy after standard fixation and processing.

Electrophysiological study was carried out on small, whole colonies which had become well established on their glass or plastic substrates. A slow flow of water was maintained through the preparation dish during the experiments to ensure that the colonies behaved as nearly as possible as in nature. Thus, temperatures in the preparation dish were kept close to those in the seawater systems at the laboratories where the animals were maintained (17–19°C at Naples, 11–13°C at Friday Harbor). A simple thermistor flow meter (Mackie *et al.*, 1983) was used to record changes in water flow velocity out of the cloacal apertures. For stimulation and recording, polyethylene suction electrodes were used. Signals were amplified and displayed on an oscilloscope or on a chart recorder. For consistency with our earlier papers on tunicate electro-

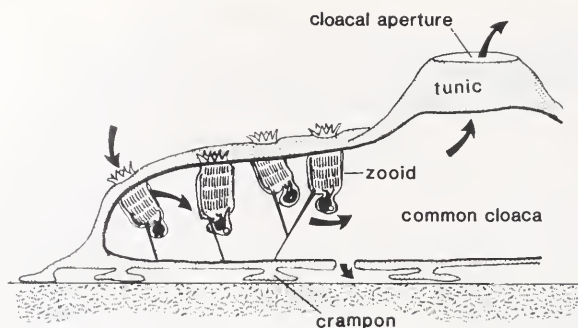


FIGURE 1. *Diplosoma listerianum*, cut-away drawing after Lahille (1890). The zooids hang by their oral siphons from the upper tunic layer and are anchored below by strands of tunic drawn up from the basal tunic layer, which is attached to the substrate by "crampons." Arrows show water flow.

physiology (Bone and Mackie, 1982) the polarity of the electrical records is arranged so that negative events go up, positive down.

General description of Diplosoma and its activities

In *Diplosoma* and other didemnids the tunic is drawn out into thin sheets—an upper sheet from which the zooids are suspended and a lower (basal) sheet which attaches to the substrate (Fig. 1). The tunic material composing these sheets is directly exposed to the seawater on both sides, and lacks an epithelial covering. A thin layer of tunic encases the zooids (depicted by Carlisle, 1953) and this continues down into an attachment strand ("stalk") which anchors the zooid to the basal tunic sheet. A retractor muscle and fine blood vessels (30 μm diam.) pass down the stalk from the zooid. It is incorrect to refer to the stalk as the retractor muscle (*e.g.*, Berrill, 1950) as it is composed primarily of tunic, and the muscle penetrates it for only a short distance. The blood vessels entering the stalk, typically four (Pizon, 1905), enter the basal sheet and run out into it, terminating in vascular ampullae. The ampullae contract and expand, pulsating rhythmically as in other ascidians, but never swell to more than 250 μm in diameter. Contrary to the arrangement in colonial styelids such as *Botryllus*, the blood vessels of different zooids are not interconnected. The vascular ampullae are responsible for the formation of 'crampons' (*ramponi*, *Wurzeln*): specialized patches of tunic material 180–240 μm in diameter by which the basal tunic adheres to the substrate. The ampullae, along with their blood vessels, may withdraw after the crampons are complete, leaving behind an attachment strand of pure tunic material. These strands are most conspicuous around the edges of the colony (Fig. 2). When elongated, they resemble the guy-ropes of a tent (Carlisle, 1961). Crampons are also present underneath the colony, roughly four per zooid stalk.

Water enters the colony through the oral siphons of the ascidiozooids. As the zooids lack atrial siphons, water passes directly out into the common cloacal cavity from which it finally exits via large cloacal apertures, which are often more than 1 mm in diameter. The exhalent water forms a plume that may rise to a considerable height above the surface of the colony. Small apertures (<150 μm) are also present in the basal tunic (Fig. 1) and water passes through them into the narrow space between the tunic and the substrate and then to the exterior. The cloacal apertures are simply holes in the tunic and should not be referred to as siphons, as they are not parts of zooids. A single large cloacal aperture may serve as the exhalent water route

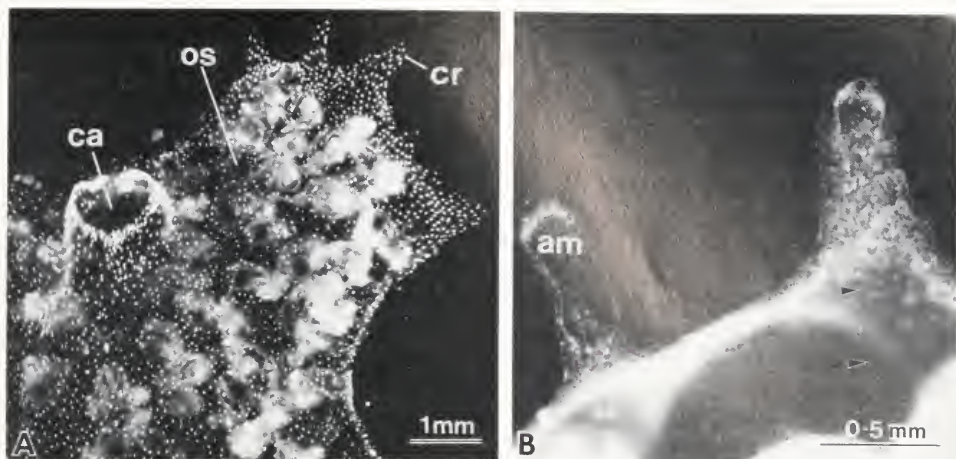


FIGURE 2. *Diplosoma macdonaldi*. A. Portion of a colony seen from above, showing a cloacal aperture (ca), crampons (cr), and zooids, some with their oral siphons (os) in focus. B. Enlargement of edge, showing two crampons, both containing vascular ampullae. The one on the left (am) is expanded, while the one on the right—which comes from another zooid—is contracted. Arrows show the blood vessel of the ampulla on the right.

for some 50 zooids. Stimulation of the tunic at any point results in slow closure of the cloacal apertures, a response discussed in detail below.

A well-maintained colony which is actively feeding and growing in undisturbed conditions tends to be flat, the stalks of its zooids very short ($<100\ \mu\text{m}$), and the common cloacal space relatively small. The blood vessels passing down the zooid stalks extend well out into the basal tunic. Around the edges of the colony these vessels push out and form crampons (Fig. 2B). In colonies which are not feeding and growing so vigorously or which have been kept in stagnant water for a few hours, the blood vessels retract and retreat up the stalks into their zooids, trailing their ampullae behind them. At the same time, the stalks elongate and are drawn out into thin strands 1 mm or more in length. Elongation of the stalks accompanies swelling of the cloacal space with exhalent water, and the whole colony expands. These changes, documented in part by Della Valle (1908, and earlier papers cited), seem to be a response to changed water conditions, but it is interesting to learn that in *Diplosoma virens* expansion and contraction are periodic events exhibited according to a diurnal rhythm (J. S. Ryland, pers. comm.).

Didemnid colonies are known to be capable of locomotion (e.g., Della Valle, 1908; Carlisle, 1961; Ryland *et al.*, 1984). The exact mechanism of locomotion has never been properly analyzed, but it involves the projection of finger-like tunic processes containing blood vessels, whose ampullae form new crampons at attachment sites ahead of the colony in the direction of movement. At the rear end of the moving colony these attachment processes, vacated by their blood vessels, are stretched out thin and eventually detach or break off. There is some evidence of positive phototaxis: Della Valle (1908) found that colonies tended to move upward in the public display tanks at Naples—which are lit from above—stopping only when they reached the surface. Carlisle (1961) found that *Diplosoma* moved sideways when illuminated from the side. Crampons once formed cannot be lifted up and moved to another site, so the movement cannot be thought of as a type of 'walking'; rather, it resembles the

motion of a tracked vehicle, a slow flowing over fixed points which presumably requires secretion of new tunic at the advancing end. The process requires further study.

The ascidiozooids of *Diplosoma* behave like solitary ascidians (reviewed by Bone and Mackie, 1982), pumping water continuously when not disturbed, and contracting their oral siphons and arresting their cilia in response to mechanical interference, as for instance with the entry of an excessively large food particle. Stronger mechanical stimulation causes retraction of the whole zooid by the retractor muscle which runs down into the proximal part of the stalk. These activities are carried on independently by the zooids. Stimulation does not cause the spread of zooid contractions or ciliary arrests across the colony. This is in marked contrast to the situation in *Bortryllus* and its relatives, where signals propagate through the colonial network of blood vessels triggering behavioral events in the zooids (Mackie and Singla, 1983).

Histology

The living tunic is soft, pliable, and transparent. The ground substance shows no regional differentiation except at the surfaces, where there is a thin (50 nm) cuticular layer comparable to the "outermost cuticle" of *Ciona* tadpole larvae (Gianguzza and Dolcemascolo, 1984), but bearing a fuzzy surface coating 200 nm thick. There appears to be no counterpart to the subcuticular zone seen in adults of this and other solitary ascidian species (De Leo *et al.*, 1981; D'Ancona Lunetta, 1983), but a layer about 200 nm deep underlying the cuticle is more densely fibrous than in other regions. Calcareous spicules are present (Carlisle, 1953) but are extremely small ($<10\ \mu\text{m}$) and far apart. Conspicuous in all parts of the tunic are the large, spherical, vacuolated cells termed "kalymmocytes" by Salensky (1892) which are probably the counterparts of the bladder cells (*Blasenzellen*) or Saint-Hilaire (1931) and the *cellules vésiculeuses* of Godeaux (1964). Pérès (1948)—one of the few authors to study post-larval *Diplosoma*—calls them "lacunae," which is clearly inappropriate, as they are cells, not spaces. Also present are cells resembling the granulocytes, morula cells, phagocytes, and other immigrant blood cells described in various tunicates by various authors. Much uncertainty surrounds the identification of such cells, but this is irrelevant to the present discussion. Bacteria are usually present in the tunic ground substance.

Of particular interest in the context of the present investigation are two cell types, both with processes interconnecting to form networks. Neither of these is clearly identifiable on the basis of previous descriptions, so they will be given new names: filopodial cells and myocytes. Filopodial cells (Fig. 3A) are restricted to the surface layer of the tunic, while the myocytes lie deeper. Filopodial cells are flattened in the plane of the surface layer, with three or more broad cytoplasmic expansions resembling neuronal growth cones, each of which subdivides into numerous fine filopodia. The filopodial cells form a fairly regular network, and are spaced out so that the filopodia just make contact. The cells termed myocytes (Fig. 3B, C) are usually bi-, tri- or multipolar, with thicker, much longer processes than the filopodial cells. Their processes show few branches, and rarely subdivide to form filopodia. They are fairly straight, and run for considerable distances through the territories of adjacent myocytes, making numerous contacts with other such processes. The myocyte layer is thick, not two-dimensional like that of the filopodial cells. The myocytes are present in all parts of the tunic but are concentrated into sphincter-like bundles around the cloacal apertures (Fig. 3C) and around the necks of the ascidiozooids. Their presence and circular orientation in these places strongly implicates them in the role of the contractile elements responsible for constricting the cloacal apertures and for pulling

in the tunic over the ascidiozooids when retracted, hence the designation "myocyte." The filopodial cells seem less likely to fulfill such a role, as they show no such concentrations around the openings, and because their processes seem too delicate to be effective as contractile elements.

Material stained with NBD-phalloidin and examined under a fluorescence microscope at 460 nm showed the myocytes as uniformly fluorescent objects, indicating the presence of F-actin (Fig. 4A). Kalymmocytes also reacted positively, but other cells in the tunic showed little response. The filopodial cells showed a very weak fluorescence, and only their thicker processes could be seen at all.

Under the electron microscope (Fig. 5), the myocytes are characterized by dense masses of rather loosely arranged fine microfilaments. True smooth muscle in ascidians by contrast shows thick and thin myofilaments arranged in strictly parallel arrays (Nevitt and Gilly, 1986). Further, using NBD-phalloidin, true muscle from the mantles of the ascidiozooids in *Diplosoma* showed a much stronger fluorescent reaction (Fig. 4B) than was apparent in myocytes in the same preparations. For these reasons, and because of their arrangement in the form of a diffuse plexus, it seems appropriate to recognize the myocytes as a new cell type distinct from conventional smooth muscle.

As noted, the filopodial cells and the myocytes lie in different layers of the tunic, and show few points of contact; therefore, while it is conceivable that the filopodial cells could represent a primitive, neuroid conduction network mediating responses of the myocytes, the likelihood of this seems remote. However, we do not know what function the filopodial cells serve.

RESULTS

The tunic pulse system: basic properties

Colonies growing in good condition on slides sometimes show no electrical activity in the tunic. Usually, however, it is possible to detect spontaneous patterns of small electrical impulses (tunic pulses, TPs) which propagate without decrement through all parts of the tunic, showing no alteration in wave form even when recorded at distances of several centimeters from the site of initiation. They can be conducted through narrow bridges of tunic less than 0.5 mm wide. They are exhibited in newly settled colonies with only two or four zooids. TPs may be evoked by tactile and electrical stimulation of the tunic as well as appearing spontaneously. Their characteristics may be summarized as follows:

Wave form. When the electrode is first attached it may be impossible to detect any signal above the noise level, as a dense plug of tunic must first fill the tip of the electrode. However, once the electrode is well attached, and usually after 30 minutes, signals can be recorded without difficulty for hours or even days. With fine suction electrodes (*ca.*, 50 μm I.D.) attached to the surface of the tunic, the signals are recorded as initially positive-going events rarely exceeding 100 μV in amplitude, with a small but long-lasting negative after-potential (Fig. 6A), and a total duration of about 2.5 s. With larger-bore electrodes the events are more symmetrically biphasic. Recordings from the inner and outer surfaces show similar TP wave forms and amplitudes. Attempts to record intracellularly from the myocytes failed, so interpretation of these extracellularly recorded events is difficult, but they are probably compound action potentials representing the summed depolarizations of many conducting elements. Somehow, the topography of the electrode attachment area converts these summed negative events into a predominantly positive-going signal. With a fine elec-

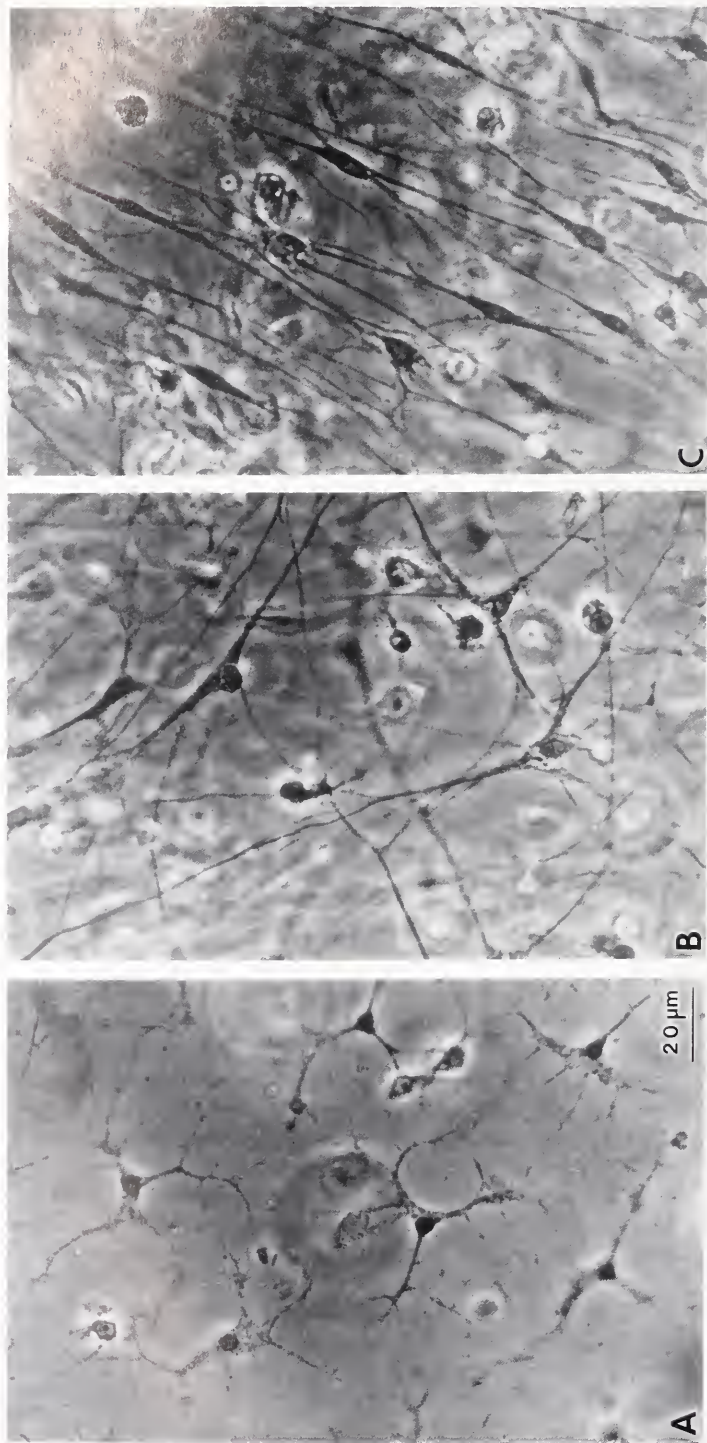


FIGURE 3. Cell networks in the tunic seen by phase contrast, all to the same scale. A. Filopodial cells, in a layer near the surface of the tunic; B. Myocytes from deeper in the tunic; C. Myocytes in circular bundle from edge of cloacal aperture.

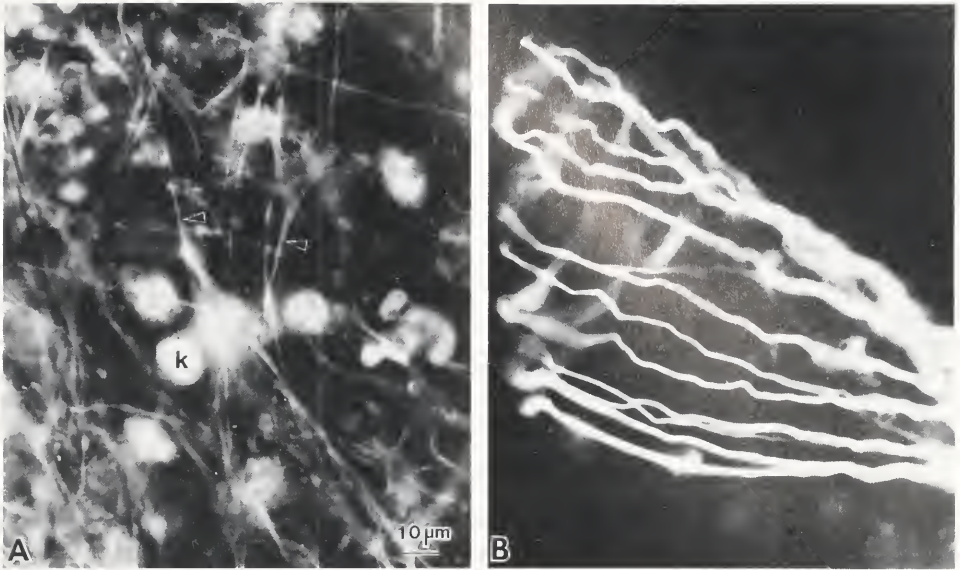


FIGURE 4. NBD-phalloidin: A. Fluorescent reaction in myocyte net (arrowheads), and in shrunken kalymmocytes (k); B. In conventional muscle from mantle wall of a zoid.

trode there would be relatively few conducting elements contributing to the signal, and they would tend to fire in synchrony so the wave form shown in Figure 6A may closely approximate the fundamental event recorded d.c. from a single cell.

Slow conduction. Conduction velocity is $1.0\text{--}1.5\text{ cm}\cdot\text{s}^{-1}$ at 19°C (Fig. 6B). No

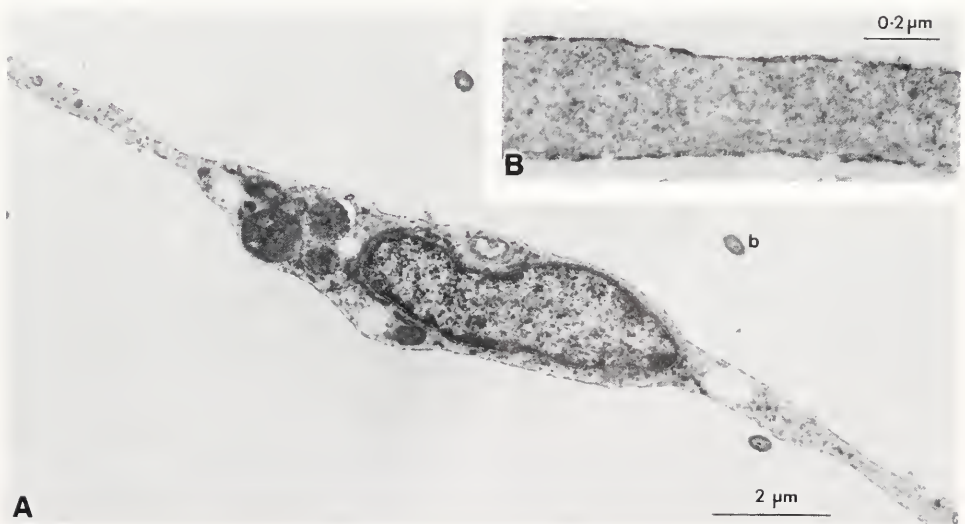


FIGURE 5. Electron micrographs of a myocyte (A) and its process enlarged (B), showing fibrillar contents. Bacteria (b) are often present in the tunic ground substance.

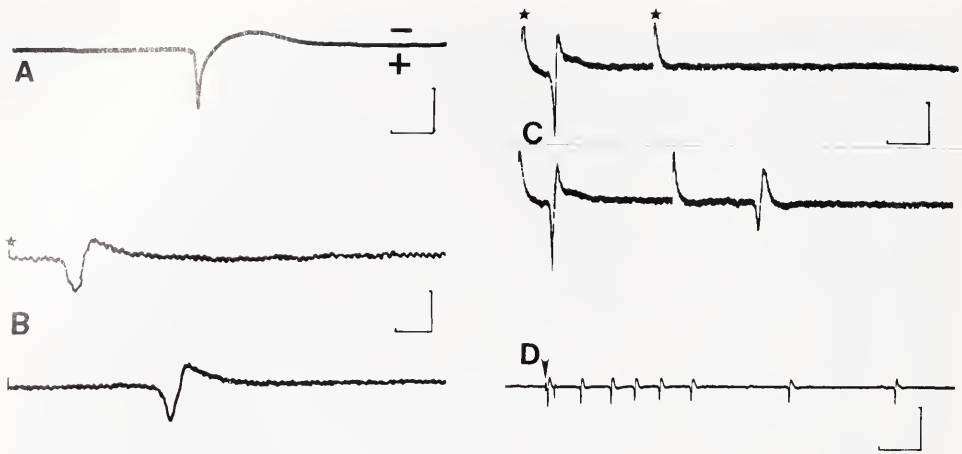


FIGURE 6. Tunic pulses (TPs). A. Spontaneous TP recorded under optimal conditions with a fine ($50\text{ }\mu\text{m}$ I.D.) extracellular suction electrode (scale bars: 1 s, $100\text{ }\mu\text{V}$). B. A TP recorded sequentially from the inside of the basal sheet of the tunic (upper trace) and from the outside of the upper sheet (lower trace) following a shock (*) on the basal sheet. Recording electrodes were 3 mm apart, conduction velocity $1.3\text{ cm}\cdot\text{s}^{-1}$ (scale bars: 100 ms, $50\text{ }\mu\text{V}$). C. With two shocks (*) 1.6 s apart, a response was elicited only to the first shock (upper trace). When the interval between shocks was increased to 1.8 s, both shocks were followed by TPs (scale bars: 0.5 s, $50\text{ }\mu\text{V}$). D. A mechanical stimulus (arrowhead) elicited a burst of TPs (scale bars: 10 s, $200\text{ }\mu\text{V}$).

significant variations in conduction velocity were observed in different parts of the tunic. Conduction time increases markedly with successive shocks. With shocks at 7 s intervals, conduction time increased by 50% of its initial value after only 6 pulses. It is not clear if increasing conduction time is due to slower conduction or to passage of impulses via less direct routes.

Long refractory period. At 19°C , the absolute refractory period was 1.6 s (Fig. 6C). In the figure, a second response was obtained with two shocks 1.8 s apart, but the amplitude of the second TP was considerably reduced, and showed a longer latency. A long refractory period would be expected if the action potential has a long duration, as proposed above.

Mechanical and electrical excitability. TPs can be evoked by pinching or pricking the tunic (Fig. 6D) or by delivering electrical shocks through a suction electrode attached to it. As with the recording electrodes, a plug of tissue must fill the tip of the stimulating electrode firmly before experiments can begin. Large shocks are needed, undoubtedly due to current shunting through the aqueous component of the tunic. Responses can usually be obtained with stimulator settings of 30–50 V, 2–5 ms, using an electrode with an internal tip diameter of about $120\text{ }\mu\text{m}$. Chemical sensitivity was not examined in detail, but the mucus and body fluids of a small keyhole limpet (species undetermined) which is the most obvious predator of *Diplosoma* colonies in the seawater system at the Stazione Zoologica had no effect on spontaneous pulse patterns recorded from the tunic. The TP system does not appear to be affected by changes in light intensity, but this aspect also needs further study.

Spontaneity. Specimens studied in as near to natural conditions as attachment of electrodes would allow showed either (a) absence of all electrical activity, (b) steady, almost metronomic pulse trains going on for periods of hours in some cases, typically with TPs 7–10 seconds apart (Fig. 7A), or (c) bursts of TPs repeated at regular inter-

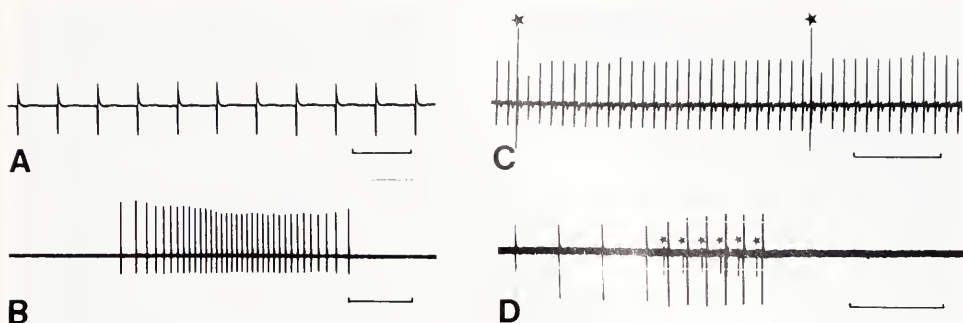


FIGURE 7. Spontaneous TP patterns. A. Steady pulse pattern (scale: 10 s). B. 'Parabolic' burst (scale: 5 min). C. Resetting of steady TP pattern by delivering shocks (*) to produce premature firing of the system. Note that the TP elicited by the shock and the one following are both of reduced amplitude (scale: 1 min.) D. Termination of steady, spontaneous TP pattern by electrical stimulation (*), causing a high frequency, artificial TP burst (6 TPs, 6 seconds apart, scale: 30 s).

vals. These sequences vary considerably, but in typical long-term burst patterns, the bursts last about 10–18 minutes (Fig. 7B), comprise 20–35 individual TPs, and are followed by 10–18 minutes of silence before the next burst. Spike frequency increases during the early part of the burst and decreases toward the end. If spike frequencies are plotted graphically, the curve approximates to a parabola. Parabolic bursting is typical of many pacemakers *e.g.*, many molluscan neurons (Strumwasser, 1968). In a preparation exhibiting a steady TP pattern, delivering a shock slightly before the next predicted spontaneous event resets the pacemaker (Fig. 7C). A steady TP rhythm can be terminated or interrupted by stimulating the preparation at a frequency greater than the rhythm frequency (Fig. 7D).

The ability to produce pulse trains and burst patterns is not restricted to any particular part of the tunic. Small pieces of tunic with no zooids in them from various parts of the upper and basal sheets produced rhythms similar to those seen in intact colonies.

Effect of elevated Mg^{2+} . TP rhythms continued unaffected in 81 mM Mg^{2+} . In 105 mM Mg^{2+} , spontaneous TP patterns ceased, but the system could still be excited electrically. In 150 mM Mg^{2+} , all TP activity ceased. These findings suggest that either conduction, contraction, or junctional transmission in the myocyte network is dependent on extracellular calcium, as magnesium ions block calcium channels (Hagiwara and Takahashi, 1967).

Effect of curare and acetylcholine. Tubocurarine chloride had no effect on the wave forms of TPs nor on their spontaneous patterns when used at concentrations up to 5×10^{-5} g·ml⁻¹ over 24 hours. Addition of acetylcholine chloride to the same final concentration had no detectable effect. These findings suggest that nerves are not involved in the tunic responses, as peripheral nerves in tunicates typically operate through cholinergic synapses (*e.g.*, Florey, 1967; Mackie *et al.*, 1974).

Electrical activity of ascidiozooids

Recordings from the zooids show ciliary arrest potentials (CAPs) like those described in numerous other tunicates (reviewed by Bone and Mackie, 1982). As reported by Mackie (1974) for another compound ascidian, *Distaplia occidentalis*, the CAP patterns of different zooids in the colony show no coordination. Attenuated

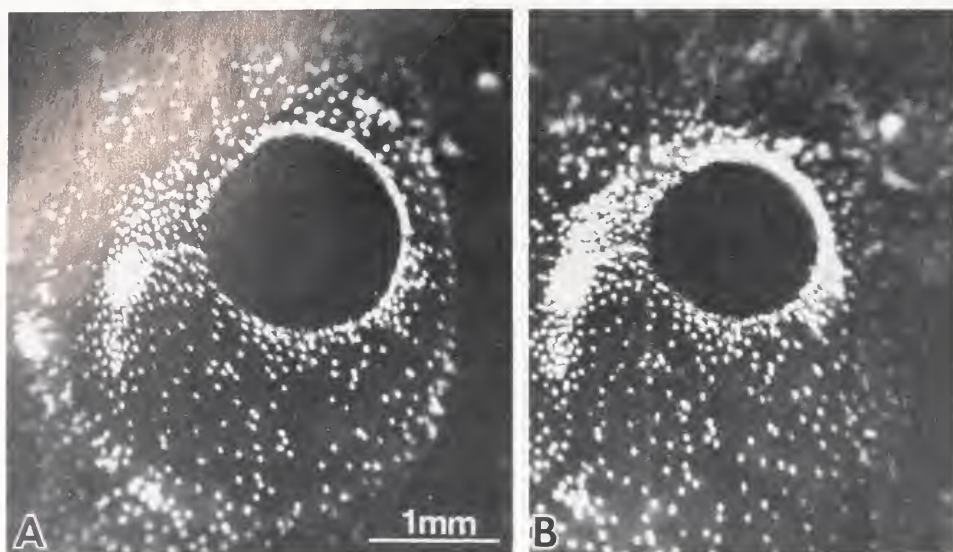


FIGURE 8. Cloacal aperture before (A) and after (B) stimulation of the tunic. Three TPs were elicited 10 seconds apart, leading to reduction of the circumference of the aperture by 17%.

CAPs can be recorded a short distance down the zooid stalk and in the upper sheet of the tunic close to the zooids; these signals are probably picked up electrotonically, rather than being conducted events.

Recordings from the vascular ampullae show small potentials similar to those recorded from the ampullae of colonial styelids and *Perophora*, and like them exhibited in a rhythm coinciding with the contractions which propel blood through the system (Mackie and Singla, 1983). Ampullae belonging to the same zooid are coordinated, but those of different zooids are not. The two ampullae shown in Figure 2B belong to different zooids and are out of phase. Cycle time is about 140 s and, as in *Botrylloides*, the potentials typically occur in doublets.

Effector correlates of tunic pulses

So far as we know, tunic pulses have no relationship to the electrical pulse patterns recorded from the zooids, and vice versa; nor do TPs seem to be involved in the locomotory process. Locomotion has been observed in colonies showing no TP patterns as well as in those showing such patterns. In fact, it seems unlikely that locomotion is controlled by any colony-wide coordinating system. The pulsatile movements of the blood vessels and vascular ampullae certainly play a part in locomotion but they are not coordinated on a colonial basis.

The only clearly demonstrable effect of TP activity is the contraction of the cloacal apertures (Fig. 8). Constriction of the aperture results in an increase in the rate of water flow through the opening. This occurs in a stepped manner, with each step corresponding to a single TP (Fig. 9). Following cessation of TPs, the aperture relaxes slowly. This effect of TPs can be observed both during experimentally induced and spontaneous TP activity, given repetitive firing at a sufficiently high frequency.

For more detailed study, given the sluggish nature of the response, it was conve-

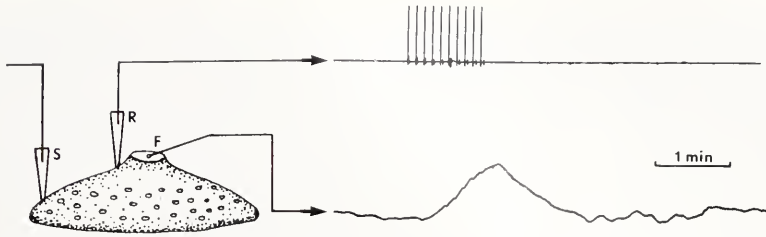


FIGURE 9. Change in rate of water flow through a cloacal aperture following stimulation of the tunic. A stimulating electrode (S) on the tunic evokes TPs, picked up with a recording electrode (R) and shown as small events following large stimulus artifacts on upper trace. Lower trace shows stepped increase in flow rate accompanying the stimulus train, recorded with a glass based thermistor flow meter (F). Following the stimulus train, flow rate returns to normal as the cloacal aperture dilates.

nient to monitor changes in the cloacal apertures visually, using a scalar eyepiece to measure diameters, from which changes in circumference could be calculated. (The myocytes are arranged in circular arrays around the openings, so changes in circumference represent length changes in the contractile tissue.) As expected, long TP bursts produce more contraction than short TP bursts at any given pulse frequency. With shocks set to evoke TPs at intervals of six seconds, summation of contractions is approximately linear until the preparation has shortened to about two-thirds of its resting length, when the curve flattens out (Fig. 10). Pulses more than about 15 seconds apart do not usually produce a summing response. It was observed that relaxation following contraction generally involves a period of hyperextension, after which the preparation returns to its resting length (Fig. 11), but no TP activity accompanies this final phase. Finally, it was shown that with long duration pulse trains at any given frequency, the preparation fails to maintain the level of contraction exhibited initially, but lengthens to a plateau level which is maintained indefinitely (Fig. 12).

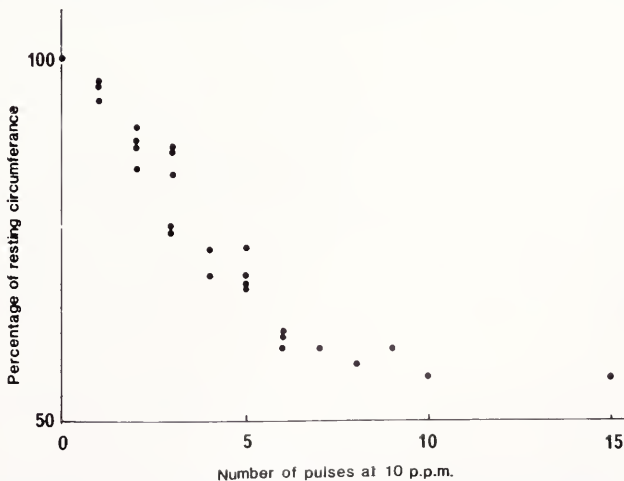


FIGURE 10. Summation of contractions during TP trains evoked by stimulation at 10 pulses per minute.

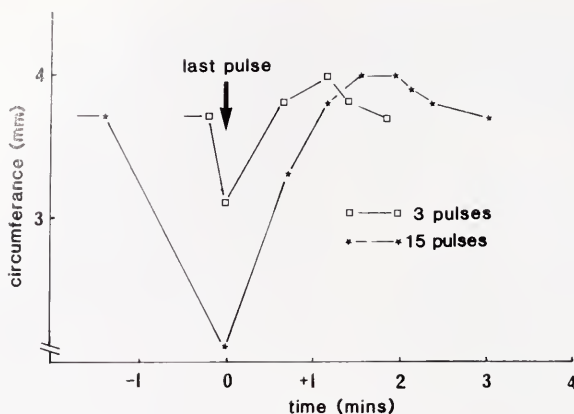


FIGURE 11. Changes in circumference of a cloacal aperture following TP trains of 3 pulses and 15 pulses, both at 10 pulses per minute.

For the experiments reported above, stimulation parameters were deliberately kept at a moderate level, so that each shock produced a single propagated TP. Stronger stimulation which causes multiple firing of the TP system, or repetition of normal stimuli at higher frequencies can produce almost complete closure of the apertures. Under these circumstances, the whole upper surface of the tunic has contracted to some extent, and the cloacal space has diminished. Therefore, although no attempt was made to quantify these observations, it seems clear that the contraction of the cloacal apertures is only part of an overall contractile response involving the whole tunic.

Re-examination of *Botryllus*

The discovery of a tunic conduction system in *Diplosoma* raised questions about our earlier results with *Perophora* and with *Botryllus* and its relatives (Mackie and

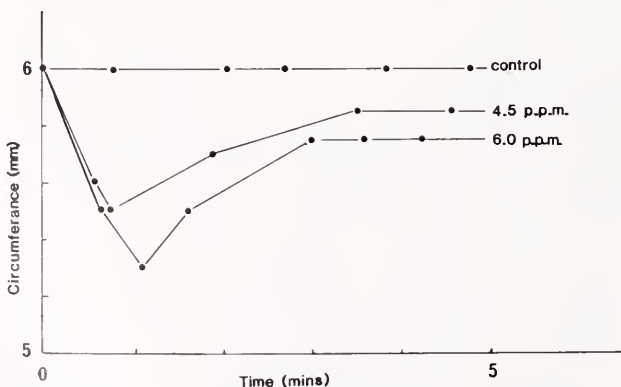


FIGURE 12. Changes in circumference of a cloacal aperture as observed over a five minute period with stimulation at two different frequencies, and with an unstimulated control. Each shock produced a single TP.

Singla, 1983), where we found that coordination of colonial activities occurred by epithelial conduction in the blood vessels connecting the zooids. It is conceivable that in these cases conduction also involves myocytes in the tunic itself. Therefore, the earlier investigation was repeated using *B. schlosseri*, which grows on the walls of the storage tanks at the Naples aquarium. The earlier results were correct. The propagated signals in *Botryllus* can be recorded only from the vascular ampullae and blood vessels. There is no sign of conduction in parts of the tunic where there are no blood vessels. It was confirmed that the blood vessel impulses ("network potentials," NPs) cause ciliary arrests in the zooids, as earlier claimed. Therefore, this NP system in *Botryllus* is distinct from the TP system in *Diplosoma*. It is interesting that *Diplosoma* has a version of the NP system, but it operates only within the confines of individual zooids, and presumably functions to coordinate the contractions of the four vascular branches which run out into the tunic from each zooid. Thus, the NP system occurs in Aplousobranchs (*Diplosoma*), Phlebobranchs (*Perophora*), and Stolidobranchs (various Botryllinae) and must be regarded as a basic ascidian action system. To date the TP system has been identified in only one family of aplousobranchs, the Didemnidae, represented in *Diplosoma*, and may be peculiar to this group.

DISCUSSION

The evidence presented here demonstrates the ability of the tunic of a didemnid ascidian to conduct all-or-none propagated impulses in response to electrical stimulation and for these signals to cause contractions of the tunic. No such findings have been reported for other species, and it seems probable that the properties of conduction and contraction are not widespread in the Ascidiacea, and may indeed prove to exist only in the family Didemnidae. The system enables the colony to control its exhalant water stream, a function performed in most ascidians at the individual zooid level, by muscles in the walls of the atrial siphons. The zooids in didemnids lack atrial siphons, and the only way of controlling water outflow is by regulating the size of the openings in the tunic (the common cloacal apertures). Therefore it seems possible that the properties of conduction and contraction in the didemnid tunic evolved in parallel with the reduction and loss of the atrial siphons of the zooids, primarily as a way of allowing the organism to control its exhalant water currents.

It is not clear exactly what benefits would be associated with the ability to regulate water flow through the colony. Strong stimulation can produce almost complete closure of the cloacal apertures, which might be advantageous in the presence of a predator. Less strong stimulation causes constriction of the apertures and produces narrow, high velocity water plumes, which rise to a greater height above the colony; this would reduce the amount of water recycled through the colony and increase advection of fresh water from the surroundings. Contractility also allows the colony to regulate the volume of water in its cloacal cavity thereby enabling it to expand or contract, an adaptation that might be put to a variety of uses. As noted earlier, *Diplosoma virens*, which possesses photosynthetic symbionts (*Prochloron*) in its tunic, expands and contracts on a diurnal basis (J. S. Ryland, pers. comm.).

We have searched in vain for evidence that the tunic conduction pathway mediates protective responses of the zooids. The majority of colonial animals have some means of coordinating their defensive responses, and this is true of ascidian colonies like *Perophora* and *Botryllus*, whose zooids are coordinated by signals transmitted through the colonial vascular system (Mackie and Singla, 1983). But *Diplosoma* appears to be an exception. Here there is no colonial vascular network and impulses propagated in the tunic conduction system seem to have no effect on the zooids.

Indeed, as Della Valle (1908) remarked in the context of locomotory behavior, the tunic has its own 'individuality,' meaning that it has a life of its own, functioning without reference to the zooids contained in it.

Regarding the cellular basis for conduction and contraction in the tunic, there can be little doubt that the cells termed myocytes are responsible for the contractions. It also seems likely that these cells conduct the electrical signals for their own contractions. The only other cells arranged in a net-like configuration—the filopodial cells—lie in a different layer of the tunic and make few contacts with the myocytes, so they are probably not the conducting elements. There is nothing inherently unlikely in the idea of a primitive contractile system which conducts its own impulses. Vertebrate cardiac muscle and many sorts of smooth muscle show this ability. However, we are hesitant to call the cells in question muscle cells because they exhibit a lower level of differentiation than true smooth muscle cells in tunicates, both in terms of their general morphology and of their ultrastructure. The term 'myocyte' seems best for these actin-loaded cells which lack thick myofilaments, are arranged in a loose network, conduct impulses very slowly, and show very long contraction latencies.

Non-muscle contractility is well developed in ascidians. Tail resorption in ascidian tadpoles involves the rapid transformation of squamous epithelial cells into tall, flask-shaped cells during which actin microfilaments become aligned in the apical (*Distaplia*) or basal (*Botryllus*) cytoplasm. Discussing these findings, Cloney (1982) states that "the caudal epidermis clearly provides the driving force in tail resorption." Sperm release in *Ciona* involves contraction of the sperm duct epithelium, again by organization of actin microfilaments. The assembly of the filaments is triggered by light (Woollacott and Porter, 1977). Microfilaments are also involved in the contractions of the vascular ampullae of colonial styelids like *Botryllus*, *Botrylloides*, and *Metandrocarpa* (DeSanto and Dudley, 1969; Katow and Watanabe, 1978; Mackie and Singla, 1983). The epithelial cells in these cases communicate via gap junctions, which presumably provide intercellular pathways for transmission of the impulses which coordinate the contractions of the ampullae. A similar mechanism may apply to the myocyte network in the tunic of *Diplosoma*, but intracellular recordings and demonstration of coupling between myocytes are required to prove this. The possibility that the myocytes communicate via chemical synapses cannot be ruled out, especially in view of the system's sensitivity to magnesium.

Thus we believe the tunic myocyte net is a system evolved *de novo* in *Diplosoma* and probably in other didemnids to bring about coordinated contractions of the exhalant water openings, thus bringing water flow under colonial control. Contractions are slow, conduction velocity is the slowest on record for any animal with the exception of hexactinellid sponges (Mackie *et al.*, 1983), the system has a limited carrying capacity in terms of impulse frequency, and it appears to fatigue very quickly. Nerves and muscles probably would allow the animal to respond with much more alacrity; however, there are no nerves or muscles in the tunic of any ascidian, so it would seem that there was no evolutionary starting point for a conventional neuro-muscular system and a completely new type of cell—the myocyte—had to be evolved, albeit as a rather inefficient substitute.

These findings emphasize the unusual versatility of the Tunicata in developing mechanisms of colonial coordination without ever using simple, direct nervous interconnections. *Diplosoma* uses an excitable myocyte network in the tunic, *Botryllus* uses its excitable vascular epithelium, the zooids in a colony of *Pyrosoma* signal to each other by responding visually to each others' bioluminescent flashes (Mackie and Bone, 1978), and salps relay signals between zooids by excitable epithelial pathways arranged in series with nerves (Bone *et al.*, 1980; Anderson and Bone, 1980). It is unlikely that these examples exhaust the list of possible mechanisms.

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BIVALVE HEMOCYANIN: STRUCTURAL, FUNCTIONAL, AND PHYLOGENETIC RELATIONSHIPS

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ABSTRACT

The hemocyanin-like molecule found in the blood of the most primitive bivalves (protobranchs) reversibly binds O₂. Its respiratory properties and its sedimentation behavior are both distinctive. Although its electron-dense image looks like that of the gastropod hemocyanins, its molecular weight differs from those of all other molluscan Hcs and is more consistent with the concept of bivalve hemocyanin as a pair of octopod hemocyanins. Bivalve hemocyanin occurs in the solemyoids as well as the nuculoids, which argues for the integrity of the Protobranchia as a natural taxon. The ancestral bivalve O₂ carrier was previously believed to be a simple intracellular hemoglobin, which is found in the less primitive Pteriomorpha. The most obvious interpretation of the present results, however, is that hemocyanin is the primitive bivalve O₂ carrier and that it was replaced by the red blood cell, which originated at least twice: once in the pteriomorph bivalves and at least once in other taxa.

INTRODUCTION

Recently Morse *et al.* (1986) reported the presence of electron dense images that resemble molluscan hemocyanins (Hcs) in the blood of two nuculoid bivalves. In addition, the denatured subunits exhibited the same anomalous behavior during sodium dodecyl sulfate polyacrylamide electrophoresis as those of some other molluscan Hcs, *viz.*, they migrated to a position corresponding to a lower molecular weight than expected from other aspects of quaternary structure (Van Holde, 1983; Ryan *et al.*, 1985). Finally, copper electrons were identified in the blood of one species by X-ray spectroscopy of sections of the auricle. Thus the evidence indicates the existence of a molecule that closely resembles molluscan Hc in the most primitive members of the class Bivalvia, which was formerly believed to use either heme proteins or no O₂ carrier at all (Mangum, 1980a; Terwilliger and Terwilliger, 1985).

This finding has considerable phylogenetic importance. First, the Hcs found in the various molluscan classes are believed to exhibit differences, albeit quite subtle ones, in quaternary structure (Ryan *et al.*, 1985). Therefore a comparison may elucidate evolutionary relationships between them. Second; it has been suggested that red blood cells (RBCs), which occur in the somewhat less primitive pteriomorphs, may represent the ancestral condition among the bivalves (*e.g.*, Mangum, 1980a). Third, if instead Hc is the primitive O₂ carrier in the class, then the RBC must have originated independently on at least two occasions, within the bivalves and in other taxa.

In the present contribution we report evidence of reversible O₂ binding, which demonstrates that the molecule in the blood of both groups (solemyoid as well as

nuculoid) of protobranch bivalves is a typical O_2 carrier, not a Hc-like precursor. We also describe additional aspects of molecular structure and respiratory function that enable comparison of the protobranch blood O_2 carrier with the Hcs found in other molluscs. Finally, we explore the implications of our findings for RBC phylogeny.

MATERIALS AND METHODS

Acila castrensis (Hinds) and *Cyclocardia* (= *Venericardia*) *ventricosa* (Gould) were collected near San Juan Island, Washington. *Yoldia limatula* (Say), *Nucula proxima* Say, and *Solemya velum* Say were purchased from commercial sources in Massachusetts.

O_2 uptake ($\dot{V}O_2$) of intact animals was determined as the depletion of O_2 in the PO_2 range 120–159 mm Hg, measured with a self-stirring polarographic electrode (Yellow Springs Instrument Co. Model 54). To prevent extraneous O_2 uptake by shell epibiota, the animals were disinfected by immersion for a few seconds in benzalkonium chloride (0.13%). Vacant shells given this treatment do not take up appreciable volumes of O_2 .

Blood was obtained by first inducing the animals to empty their mantle cavities and then prying open the valves about 1 mm or less with a jeweler's screwdriver. The valves were reflected backwards about 270° and the animals placed in a small funnel draining into an Eppendorf tube. When the blood ceased to drain, additional volumes were obtained by centrifuging the animals at a very low speed. After repeated problems with apparent proteolysis during sedimentation analysis, we collected the blood of *Y. limatula* by draining it directly into a mixture of protease inhibitors, which appeared to enhance the stability of the preparation. About 1 ml blood was drained into a 60 μ l solution containing 30 pg leupeptin, 30 pg pepstatin A, and 3 μ moles phenylmethylsulfonyl fluoride.

The gills of *Y. limatula* were dissected and extracted with 0.5% Na_2CO_3 . The extract was diluted by 10% with pyridine, reduced with a few grains of sodium dithionite, and, due to its very small volume, examined with Zeiss micro- and Hartridge reversion spectrometers (Mangum and Dales, 1965).

The bloods were centrifuged and immediately prepared for electron microscopy (Zeiss EM-109) by negative staining with uranyl acetate (Mangum *et al.*, 1985; Morse *et al.*, 1986). In the present investigation the blood was diluted with 0.05 M Tris maleate buffer + 10 mM $CaCl_2$ (pH 7.63) by factors ranging from 1:9 to only 1:50, because its appearance suggested a low Hc concentration. The sample and the stain were applied to the grid with an atomizer.

O_2 binding was determined on fresh (never frozen) blood samples from *A. castrensis*, *S. velum*, and *Y. limatula* by the cell respiration method (Mangum and Lykkeboe, 1979). Due to the size of the *N. proxima* individuals (2–5 mm), it was necessary to stockpile frozen material until the requisite volume (300 μ l) accumulated. About half of the material on which the measurements were performed had been frozen. The samples were diluted by 10% with Tris maleate (final concentration 0.05 M) buffered seawater (32‰) containing commercial yeast cells. An attempt to first concentrate *A. castrensis* blood by membrane filtration was unsuccessful due to precipitation of some of the material, which was also noted during the O_2 binding measurements. No precipitation of the *Y. limatula*, *S. velum*, or *N. proxima* material was observed.

The O_2 affinities of heme proteins are often concentration dependent. Because most experimental procedures require dilute solutions, the results do not accurately reflect *in vivo* values. To obtain physiologically meaningful data for the branchial

heme protein of *Y. limatula*, O₂ binding in the present investigation was also determined using whole gills, dissected intact. An absorption spectrum of the bathing medium indicated that there was no loss from the gills during the measurement. It was necessary to modify the cell respiration procedure because the method requires that the rate of free O₂ depletion be linear. This is achieved by lowering the PO₂ with particles such as isolated mitochondria or yeast cells which are so small that the diffusion distance is not limiting. If the O₂ uptake of whole unperfused gills had been allowed to make an appreciable contribution to total O₂ uptake, this condition would clearly have been violated and the apparent O₂ affinity would have been erroneously low due to an extraneous departure from linearity. The problem was circumvented by first determining the $\dot{V}O_2$ of the gills and then adding large numbers of yeast cells so that yeast $\dot{V}O_2$ was more than 10 times gill $\dot{V}O_2$. The cell respiration method also requires that the rate of O₂ depletion be slow enough to permit equilibration of the electrode at any PO₂. If this condition had been violated, the result would have been an erroneously high O₂ affinity, because an apparent oxygenation state would have coincided with a PO₂ that actually had obtained earlier, at a higher oxygenation state. The possibility was eliminated by ascertaining equilibration under the following experimental conditions: using the Hc of the crab *Cancer magister*, whose respiratory properties are well known (e.g., Graham *et al.*, 1983), the rate of O₂ depletion was increased until an erroneously high O₂ affinity was obtained. The period for depletion of free O₂ from 100 to 0% air saturation was considerably less than 25 s. In the measurements on gills, much longer periods (87–233 s) were employed.

Absorption spectra of the medium and of fresh Hcs were determined with Beckman DK-2A and Varian 2200 spectrophotometers. To eliminate light scatter, the Hc samples were first diluted with dissociating buffer (0.05 M Tris HCl, pH 8.95 + 0.05 M EDTA) by 50 to 97% depending on color intensity.

All sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with scanner optics. Wavelengths in the vicinity of the Hc bands at 280 and 345 nm were used. Temperature was controlled to <0.1°C. Sedimentation coefficients were measured from the midpoints of the well-defined boundaries and corrected to $S_{20,w}$ in the usual way. The sedimentation equilibrium experiment was conducted at 1500 rpm, using the heavy J rotor. Equilibrium was attained when no difference could be noted between scans approximately 6 h apart. After equilibrium the rotor was accelerated to 6000 rpm and a baseline recorded approximately 4 h later.

RESULTS

O₂ uptake

Intact individuals of *Acila castrensis* take up O₂ measurably, but $\dot{V}O_2$ is more than two orders of magnitude lower than in the heterodont *Cyclocardia ventricosa* (Table I), which was collected from the same bottom on the same occasion and held in the laboratory in the same container for the same period. The difference in body size can account for only a small fraction of the difference in O₂ uptake. Moreover, $\dot{V}O_2$ is also orders of magnitude lower in *A. castrensis* than in the perimorph *Noetia ponderosa*, a much larger animal measured at a slightly lower temperature (Table I).

Absorption

The dissociated subunits of the four bivalve Hcs absorb at 280 and 345 nm (Fig. 2). Other molluscan Hcs absorb in the same regions (Nickerson and Van Holde,

TABLE I

Oxygen uptake in bivalves with specialized oxygen carriers

Species	O ₂ carrier	$\dot{V}O_2$ (μ l/g dry wt-h)	Dry wt. (mg)	Temp. (°C)	Source
<i>Acila castrensis</i>	Hc	2.0 $\pm$ 0.9 (6)	107.9–122.8	11.5	present data
<i>Venericardia ventricosa</i>	extracellular Hb	269.5 $\pm$ 15.5 (6)	33.5–34.9	11.5	present data
<i>Noetia ponderosa</i>	intracellular Hb	148	5 $\times 10^3$	10	Deaton and Mangum, 1976
<i>Glycimeris nummaria</i>	intracellular Hb	ca. 49.5 ^a	2.7 $\times 10^3$	+16–20	Kruger, 1957

^a Converted from original data assuming that dry wt. = 20% wet wt. Mean $\pm$ SE (n).

1971). R. C. Terwilliger kindly communicated data for polyplacophoran Hc, which have not been reported in the literature. Observations on the Hc of the chiton *Chaetopleura apiculata* were also made together with those reported here. The peak at 345 nm disappears in the presence of sodium borohydride (*e.g.*, Fig. 1).

Extracts of the gill of *Y. limatula* clearly form a pyridine hemochromagen with absorption bands at 542 and 556 nm. High concentrations of red granules also were observed in the nerve ganglia and connectives of this species but not in *A. castrensis* or *N. proxima*, which appeared to lack branchioglobin (Bb) as well. However, a seawater extract of the whole bodies of *N. proxima* appeared to form a pyridine hemochroma-

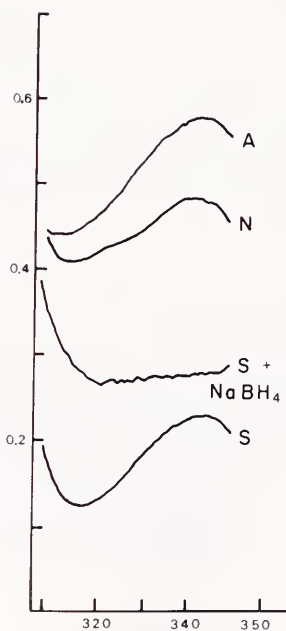


FIGURE 1. Absorption spectra of protobranch Hcs. (A) *Acila castrensis*, (N) *Nucula proxima*, and (S) *Solemya velum*. Abscissa is wavelength in nm and ordinate is absorbance.

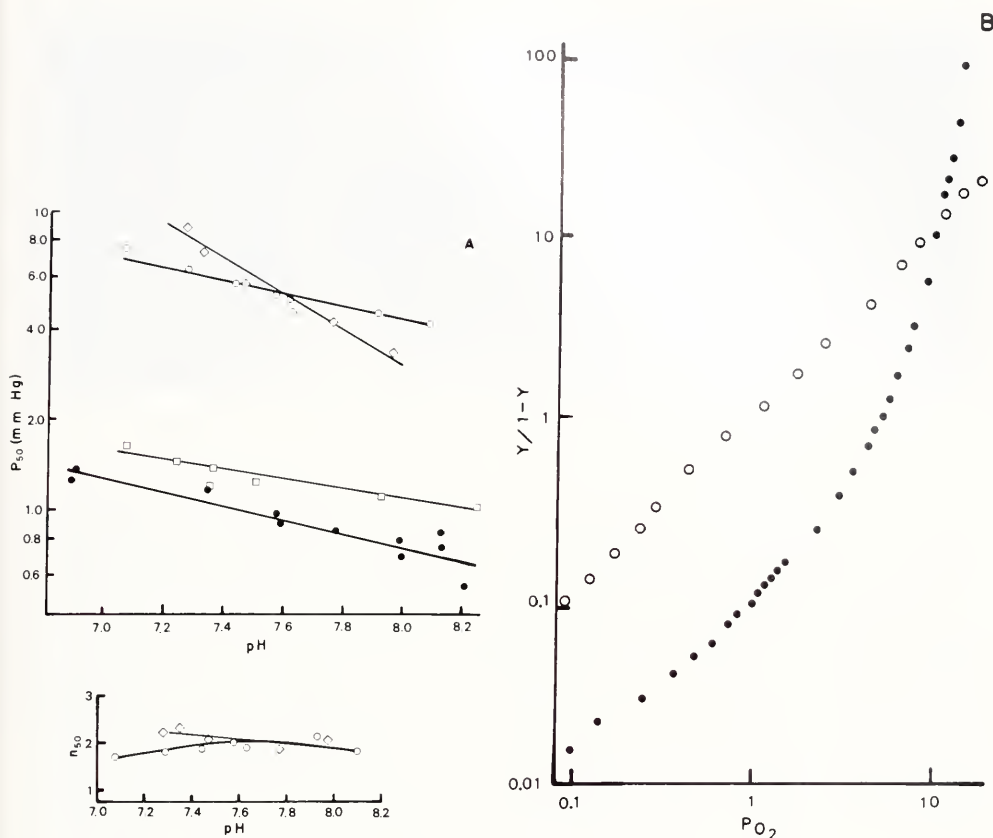


FIGURE 2. A. pH dependence of O_2 binding by *Acila castrensis* (●), *Nucula proxima* (□), *Solemya velum* (◇), and *Yoldia limatula* (○) Hcs. 20°C, 0.05 M Tris maleate buffered blood. B. Hill plot of O_2 equilibrium of *Yoldia limatula* (●, pH 8.10) and *Acila castrensis* (○, pH 7.78) Hcs.

gen, although the visual observation could not be confirmed even by microspectroscopic observation due to the very small volume (*ca.*, 10 μ l) obtained.

O₂ binding of the bloods

Unlike other Hcs, *A. castrensis* Hc binds O_2 non-cooperatively (*e.g.*, Fig. 2). The Hill coefficient (n_{50}), which is independent of pH, is $1.03 (\pm 0.03 \text{ SE}, n = 11)$. Among the Hcs, *A. castrensis* also has an unprecedentedly high O_2 affinity (Fig. 2), thus resembling tissue O_2 carriers more than most blood O_2 carriers. Unlike tissue O_2 carriers, however, *A. castrensis* Hc has a small but significant normal Bohr shift. The slope of the regression line describing the data in Figure 2, or $\Delta \log P_{50} / \Delta \text{pH}$, is $-0.23 (\pm 0.08 \text{ 95\% C.I.})$. HcO_2 binding in *N. proxima* (which belongs to the same family as *A. castrensis*) is also non-cooperative ($0.94 \pm 0.09 \text{ SE}; n = 7$) and it has a similar Bohr shift ($-0.16 \pm 0.07 \text{ 95\% C.I.}$) although its O_2 affinity is somewhat lower. The Hc of *Y. limatula* (which belongs to a different family) has a much lower O_2 affinity, though still fairly high for a molluscan Hc, and it is moderately cooperative (Fig. 2). Its Bohr shift is indistinguishable from that of the other nukuloid Hcs (-0.24 ± 0.05).

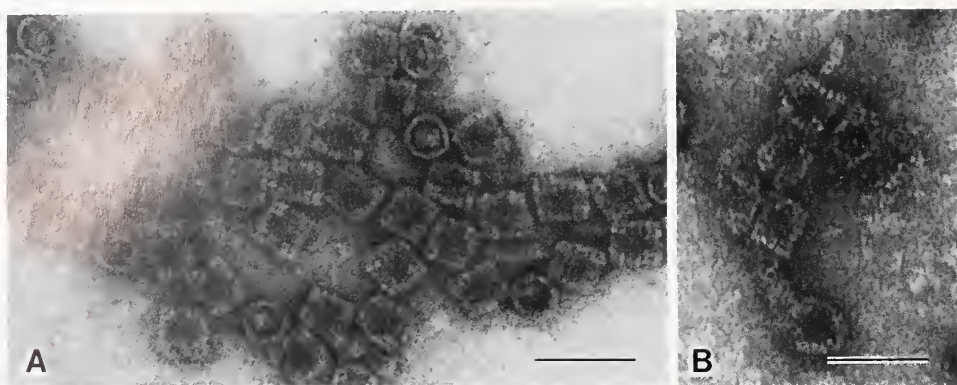


FIGURE 3. Electron micrographs of bivalve Hcs. A. *Yoldia limatula*. B. *Solemya velum*. Scale bar 50 nm.

The Hc of *S. velum* (which belongs to a different order) resembles *Y. limatula* Hc in terms of O_2 affinity and cooperativity, but its Bohr shift is much larger (-0.61 ± 0.17).

O_2 carrying capacity of the bloods ($HcO_2 + O_2$) was estimated from absorbance at the active site, using the extinction coefficient for *Busycon* Hc (Nickerson and Van Holde, 1971). At 11.5°C and 32‰ salinity the value for *A. castrensis* blood is 1.05 ml/100 ml, for *S. velum* is 1.00 ml/100 ml, and for one sample from *Y. limatula* is 0.96 ml/100 ml. *N. proxima* blood, which is much bluer than the others, carries 2.85 ml/100 ml. The figure for *A. castrensis* should be regarded as low due to precipitation in the sample. However, a similarly low figure (0.76 ml/100 ml) for another sample from *Y. limatula* was obtained from integrals of the curves describing deoxygenation (procedure detailed by Mangum and Burnett, 1986). Moreover, the difference between *N. proxima*, *S. velum*, and *Y. limatula* cannot be due to starvation of the latter two in the laboratory (which, in fact, has not been reported for molluscan Hcs) since they were held for the same period (<2 days).

O₂ binding by gills

Two determinations of O_2 binding by intact *Y. limatula* gills, which should provide physiologically meaningful information, gave P_{50} values of 0.43 and 0.46 mm Hg and n values of 0.98 and 1.02 (20.3°C, ambient pH 8.01).

Electron microscopy

Since *N. proxima* is so closely related to *A. castrensis*, the small amount of material available was used for other purposes. The shapes of *S. velum* and *Y. limatula* Hcs (Fig. 3) are indistinguishable from that of *A. castrensis* Hc, which was described earlier (Morse *et al.*, 1986). All three molecules are six-tiered cylinders and, like many gastropod Hcs, appear as circles in top view and as squares in side view (see Ghiretti-Magaldi *et al.*, 1979, van Bruggen *et al.*, 1981). They lack the "belt," or unequal spacing of the six tiers, found in one species (van der Laan *et al.*, 1981). The width (31 nm) of the *Y. limatula* squares appears to be slightly but significantly ($P < .001$ according to Student's t test) smaller than that of 12 tiered cylinders found in the blood of the gastropod *Busycon canaliculatum*. These dimensions were determined by mixing a small volume of *B. canaliculatum* blood with a large volume of *Y. limatula*

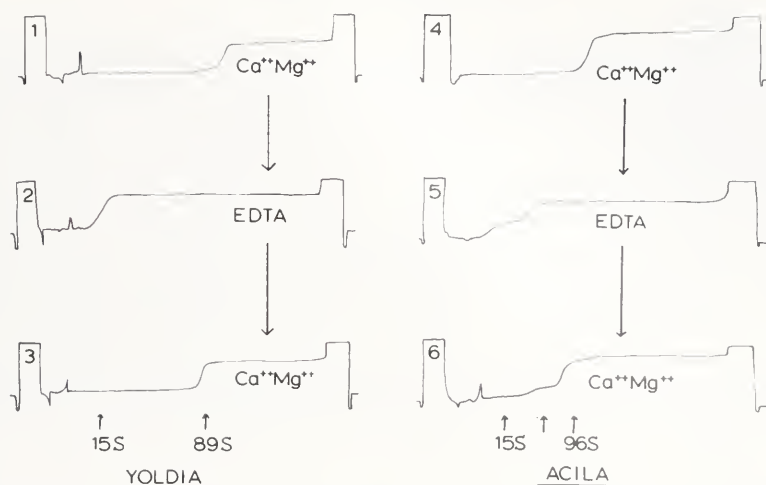


FIGURE 4. Scanner traces showing dissociation and reassociation of bivalve Hcs at pH 7.65, 20°C. *Yoldia limatula*: (1) in 0.05 M Tris-HCl, 50 mM MgCl_2 , 10 mM CaCl_2 ; (2) dialyzed against 0.05 M Tris-HCl + 10 mM EDTA; (3) dialyzed back again against the original buffer. *Acila castrensis*: (4) as in 1; (5) as in 2; (6) as in 3. In 5 and 6 the middle boundary sediments at about 55S.

blood and then measuring the width of all (24) of the 12-tiered cylinders observed and a sample of 100 6-tiered cylinders. The bivalve circles have a five-fold rotational symmetry and a collar and a cap. When dissociated to halves, the molecule looks like a three-tiered rectangle, which absorbs more stain at one end than the other, and as circles, only some of which have collars and caps (see Fig. 3 in Morse *et al.*, 1986). The images of half molecules, which have also been described for *Helix* Hc, are believed to reflect the absence of collars and caps at the broken surfaces (van Bruggen *et al.*, 1981). Like gastropod Hcs, the bivalve squares are about 35 nm long.

Physical characterization

Sedimentation velocity experiments with *A. castrensis* and *Y. limatula* Hcs were performed at room temperature and under a variety of solvent conditions. The results are summarized as follows:

(1) In 0.05 M Tris-HCl buffer (pH 7.65) containing 50 mM CaCl_2 and 10 mM MgCl_2 , both Hcs exhibited single, sharp boundaries. The sedimentation coefficients ($S_{20,w}^\circ$), when corrected to standard conditions and extrapolated to zero Hc concentration, were 95.8 for *A. castrensis* Hc and 88.8 for *Y. limatula* Hc.

(2) When the two Hc solutions were dialyzed exhaustively against 10 mM EDTA in 0.05 M Tris-HCl (pH 7.65), they behaved differently (Fig. 4). *Y. limatula* Hc dissociated completely to yield a single boundary with $S_{20,w} = 15.9\text{S}$. Under the same conditions *A. castrensis* Hc showed incomplete dissociation, yielding two components with $S_{20,w} = 54$ and 18S. Attempts at reassociation also gave quite different results. Upon dialysis of the Tris EDTA treated material back to Tris $\text{Ca}^{+2} + \text{Mg}^{+2}$, *Y. limatula* Hc quantitatively reassociated to the 89S component. Under the same conditions only partial reassociation could be attained with *A. castrensis* Hc.

(3) When *Y. limatula* Hc was dialyzed against a series of dilutions of the Tris buffer in which the divalent cations were reduced to $1/10$, $1/20$, $1/40$, and finally $1/100$ of

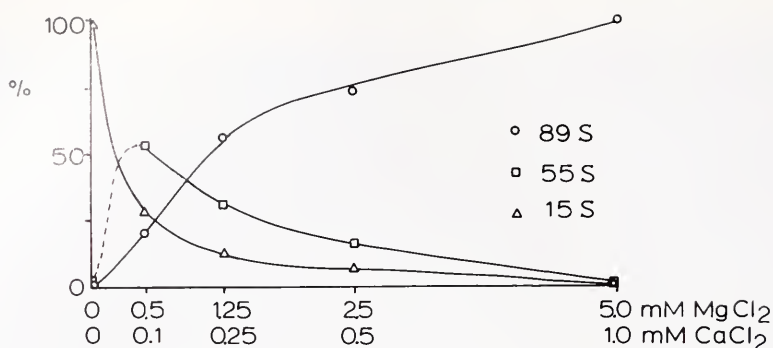


FIGURE 5. Relative amounts of *Yoldia limatula* Hc of three aggregation states when equilibrated to 0.05 M Tris buffers (pH 7.65; 20°C) containing varying amounts of MgCl₂ and CaCl₂ (see text for details).

their original concentrations, the resultant dissociation yielded a mixture of three components: the 89S Hc (the whole molecule), another with a sedimentation coefficient of 55S (probably a half molecule), and a third with a sedimentation coefficient of about 15S (Fig. 5). The present data do not indicate just what multiple of the polypeptide chain this smallest product represents, but the sedimentation coefficient corresponds to that of the dissociation product in the presence of EDTA.

The reversible dissociation behavior of *Y. limatula* Hc strongly suggests that, like *Octopus* Hc (Van Holde and Miller, 1985), it is composed of a single type of subunit. In contrast, the incomplete reassociation of *A. castrensis* Hc is more like that of other molluscan Hcs (Van Holde and Miller, 1982). Furthermore *Y. limatula* Hc, like *Octopus* Hc, dissociates in the presence of EDTA at a much lower pH than normally required for other molluscan Hcs. Although divalent cation levels must be reduced to extremely low levels before dissociation begins, *Y. limatula* Hc dissociates at pH 7.65, which is probably close to the physiological value. In all likelihood, at higher pH it would dissociate at higher divalent cation levels.

DISCUSSION

Respiratory properties and their relationships to protobranch biology

Allen (1978) suggested that protobranch bivalves are able to exist with their small and, in his view, relatively inefficient feeding organs because they have low metabolic rates. The present findings support his suggestion, at least with respect to aerobic metabolism. However, we should point out that, relative to bivalves that both use the gill as a filter-feeding organ and lack an O₂ carrier, the branchial surface area is also small in the pteriomorph *Noetia ponderosa*, whose feeding has not been investigated and whose VO₂ is not especially low (Mangum, 1980a). Almost certainly VO₂ is influenced by other factors in addition to feeding efficiency. While it is believed that conventional feeding in *Solemya* is supplemented or perhaps even supplanted by a symbiotic relationship with chemoautotrophic bacteria (Cavanaugh, 1980, 1983; Felbeck, 1983; Doeller, 1984; Fisher and Childress, 1984; Reid and Brand, 1986), no sign of bacteria can be found in electron micrographs of the gills of protobranchs such as *A. castrensis* (mentioned by Reid and Brand, 1986) and *Y. limatula* (M. P. Morse, unpub. obs.).

The uniformly normal Bohr shift of the bivalve Hcs resembles those of polyplacophoran and cephalopod Hcs. Gastropod Hcs have either reversed Bohr shifts (prosobranch), a combination of reversed and normal Bohr shifts (prosobranch and pulmonate), or none at all (opisthobranch). As indicated above, the extremely high O_2 affinity and lack of cooperativity of *A. castrensis* Hc is unique. The moderate cooperativity and O_2 affinity of *S. velum* and *Y. limatula* Hcs are common among the molluscs, although examples of much greater cooperativity are known (Mangum, 1980b). O_2 carrying capacity appears distinctively low, at least in *S. velum* and *Y. limatula*, but typical of molluscan HcO_2 transport systems in *N. proxima*. Why the nukuloids, with such similar respiratory and cardiovascular systems, should have Hcs with such different respiratory properties remains to be elucidated.

The anatomical relationships between the protobranch O_2 carriers also are intriguing. At least in *Y. limatula* and *S. velum*, O_2 must move from the environmental source into the heme protein-containing branchial epithelium. From there the O_2 moves into the Hc-containing blood, where it is carried by convection to the metabolic sink. But in both species the O_2 affinity of the branchioglobin (Bb) is higher than that of the Hc (see Doeller *et al.*, 1983, for values for *S. velum* Bb). The arrangement violates the fundamental design principle of an O_2 transfer system, which mandates the highest O_2 affinity in the compartment most remote from the environmental source. Bb must actually be a barrier to O_2 influx as long as it is not fully oxygenated.

The physiological question is complicated by uncertainty surrounding the function of bivalve Bb and other tissue heme proteins. Doeller (1984) suggested that *S. velum* Bb transports sulfide to the chemoautotrophic bacteria in the gills; the sulfide-oxidizing bacteria are believed to serve as key components of a newly discovered mode of animal nutrition. As pointed out by Dando *et al.* (1985), this function does not preclude the possibility of others, such as facilitated diffusion or O_2 storage.

We noted that the period from onset to completion of nonlinearity of O_2 uptake by *Y. limatula* gills (in the absence of yeast cells) was only 162 s. This period includes both the diffusion-limited and BbO_2 -supplied components. The molecule cannot be an O_2 store of significant longevity. We suggest that BbO_2 carrying capacity also be considered in the continuing debate on functions of tissue heme proteins.

Structural properties

The sedimentation coefficients observed for the bivalve Hcs are surprisingly low. The Hcs that would seem to resemble bivalve Hc in shape are the six-tiered cylinders found in the prosobranch whelk *Kelletia* and the pulmonate snail *Helix* (van Bruggen *et al.*, 1981). Opisthobranch and the other prosobranch Hcs studied tend to form larger aggregates or have special features such as unequal spacing of the six tiers. Cephalopod and polyplacophoran Hcs are three-tiered cylinders.

As Table II shows, however, almost all reliable measurements of the sedimentation coefficients of the gastropod six-tiered multiple yield values of $S_{20,w}^\circ$ between 100 and 105S. We were struck by the value for *Y. limatula* Hc, which is 10–15% lower. A lower value might be explained by either a looser quaternary structure, greater hydration, or a lower molecular weight. The molecular weight obtained from Figure 6 depends on the value assumed for the partial specific volume (v) (see Van Holde, 1985). Unfortunately we have neither an experimentally determined value for v for *Y. limatula* Hc nor an amino acid composition, from which it might be estimated. Values reported for molluscan Hcs range from about 0.73 (gastropod) to 0.74 (cephalopod). The former yields a molecular weight of 6.5×10^6 and the latter yields 6.8×10^6 . Either is much lower than the values reported for the gastropod 100–105 S Hcs (Table II).

TABLE II

Comparative properties of native hemocyanin molecules of bivalves and gastropods

Species	$S_{20,w}^{\circ}$ (svedbergs)	M (g/mol $\times 10^6$)	$\bar{v}$ (cm ³ /g)
Class: Gastropoda ¹			
<i>Archachatina marginata</i>	102.3	9.1	—
<i>Buccinum undatum</i>	101.1	9.0	—
<i>Busycon canaliculatum</i>	103.2	8.8	.727
<i>Helix pomatia</i> (α)	104.3	8.7	.727
<i>Helix pomatia</i> (β)	105.8	9.0	—
<i>Murex trunculus</i>	102.7	8.9	—
<i>Paludina vivipara</i>	102.5	8.7	—
<i>Pila leopoldvillensis</i>	101.2	8.7	—
Class: Bivalvia ²			
<i>Acila castrensis</i>	95.8	—	—
<i>Yoldia limatula</i>	88.8	6.5–6.8	—

¹ Data from Van Holde and Miller (1982). Original references are given therein.² Present data. The value of M for *Yoldia* hemocyanin depends upon whether a value of 0.73 (as for gastropods) or 0.74 (as for cephalopods) is assumed.

The data are more consistent with the concept of bivalve Hc as a pair of cephalopod Hcs. A pair of polyplacophoran Hcs would have a much higher molecular weight (Ryan *et al.*, 1985; Herskovits *et al.*, 1986). The cephalopod 51–60S particles, however, do not pair to form six-tiered cylinders. If they did, they might give rise to a particle with a sedimentation coefficient of about 90S and an electron-dense image much like that of *Y. limatula* Hc. If we assume that native *Y. limatula* Hc is a 20-mer of polypeptide chains, like other 6-tiered molluscan Hcs, then the chain weight must be approximately 3.4×10^5 . This is very close to the weight of octopod chains

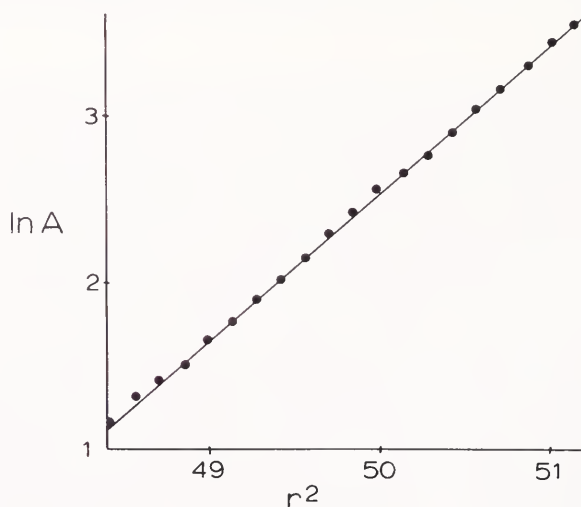


FIGURE 6. Determination of molecular weight of *Yoldia limatula* Hc by sedimentation equilibrium. A represents concentration in arbitrary units of $A_{345 \text{ nm}}$; r is the distance from center of rotation.

(Gielens *et al.*, 1986; Lamy *et al.*, 1986), and substantially smaller than that of gastropod chains ($4.0\text{--}4.5 \times 10^5$). Such a conclusion seemingly contradicts the observation that bivalve chains (Morse *et al.*, 1986) run more slowly than cephalopod chains (Van Holde and Miller, 1982) on SDS gels. However, SDS gel electrophoresis is notoriously unreliable for glycoproteins such as Hcs. It has been frequently reported that estimates of subunit molecular weight are in substantial error for these proteins.

On the basis of the available information we suggest that the bivalve Hcs may resemble octopod Hcs in having a small (relative to other molluscan Hcs) subunit, but share with gastropod Hcs the capacity to associate to 20-mers. This conclusion is supported by the apparently smaller width of the bivalve cylinders. In possible contradiction, however, we should mention that Ellerton and Lankovsky (1983) reported a 26–30 nm wide and 28–34 nm long Hc in the primitive archaeogastropod *Haliotis iris*.

Phylogeny of the molluscan Hcs

The most recent discussions of molluscan phylogeny suggest two major phyletic lines leading from the postulated ancestor, which is in turn descended from an acoelomate animal at the turbellarian-nemertine level of organization (Runnegar and Pojeta, 1986; Salvini-Plawen, 1986). One of these phyletic lines is an aplacophoran-polyplacophoran lineage and the other leads through the monoplacophorans to the gastropods, the rostroconch-bivalve-scapopods, and the cephalopods (Fig. 7). In view of the equally recent conclusion that the nemertines are, in fact, descendants of an annelid-like coelomate (and, we suggest, RBC-containing) animal (Turbeville and Ruppert, 1986), the condition of the coelom in the ancestral mollusc probably should be reconsidered. Regardless, the present findings emphasize the importance of ascertaining the properties of O_2 carriers (if any) in the poorly known molluscan classes such as the monoplacophorans and the scaphopods. We also look forward to the results of the sedimentation equilibrium studies of archaeogastropod Hc which were underway at the time of Ellerton and Lankovsky's 1983 report. The elucidation of the structures of the Hcs (if any) in these three groups and also additional members of other molluscan groups may have important implications for molluscan phylogeny. In our view, the detail available at present does not permit a very confident conclusion concerning the evolutionary relationships of the molluscan Hcs.

Origins of the red blood cell and its simple hemoglobins

If the status of postulated transitional group is disregarded, there is some consensus among molluscan systematists concerning the ancestry of the bivalves. Along with the Gastropoda, Scaphopoda, and Cephalopoda, the class Bivalvia is believed to be descended from the Monoplacophora (Cox *et al.*, 1969; Newell, 1969; Stasek, 1972; Pojeta, 1978; Runnegar, 1978; Runnegar and Pojeta, 1986; Salvini-Plawen, 1986). There is considerable disagreement, however, on the relationships of the different groups of bivalves. Newell (1969) described six subclasses and assigned the solemyoids and the nuculoids to separate ones. However, Allen (1986) argued for the integrity of the Protobranchia as a subclass that includes all bivalves with simple, pectinate gills and described only one other subclass, the Lamellibranchia. Our findings strongly support Allen's (1986) view. While we cannot provide evidence for the absence of Hc in all lamellibranchs, we can provide evidence of the absence of any O_2 carrier in the blood of one pteriomorph (*Modiolus demissus*) and two heterodonts (*Crassostrea virginica* and *Rangia cuneata*): When measured with a Lexington In-

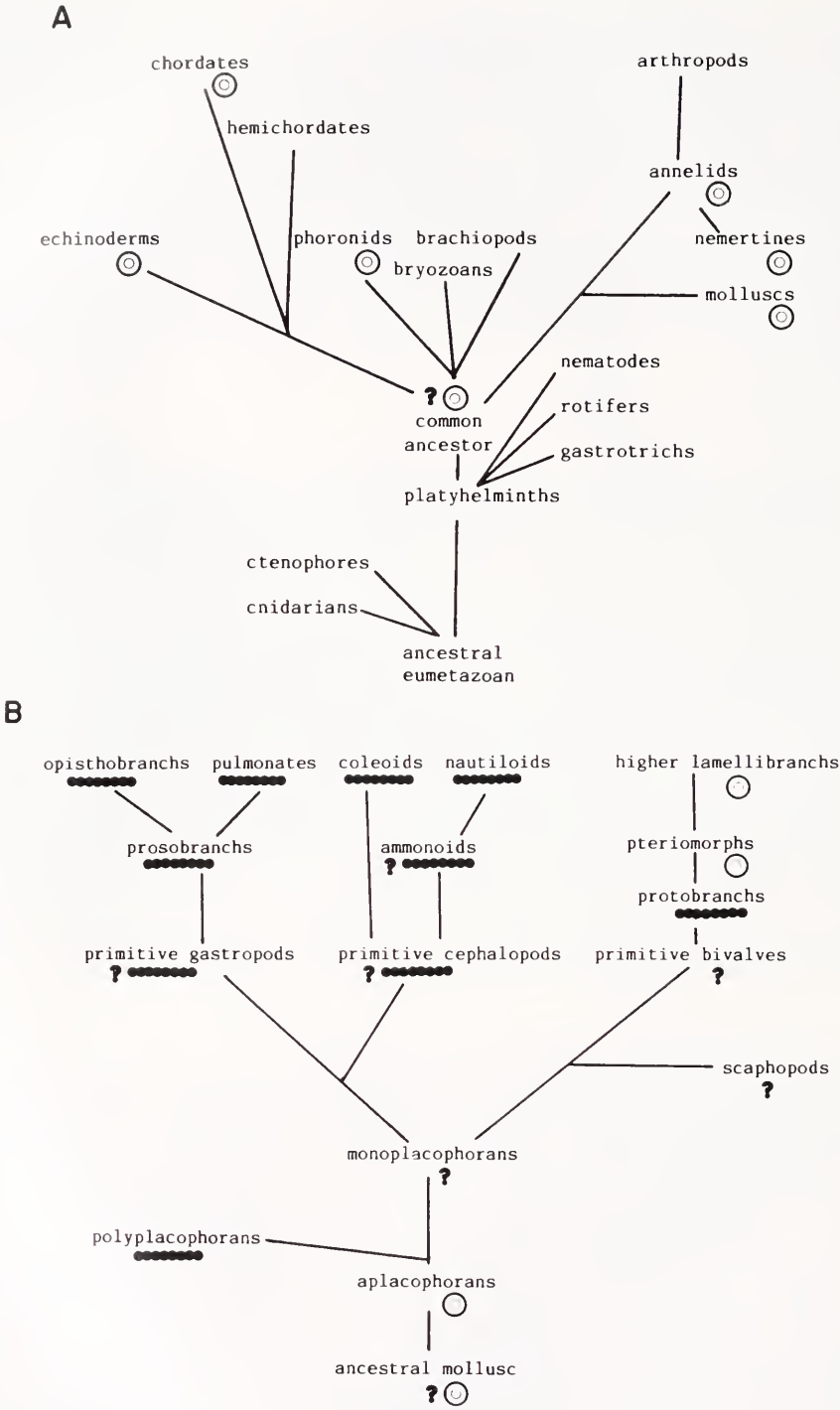


FIGURE 7. Phylogeny of: A. The red blood cell; B. The molluscan O₂ carriers. (⊗) symbolizes RBCs and (.....) symbolizes molluscan Hc. Question mark indicates uncertainty.

struments Co. analyzer, the total O₂ contents of these bloods did not differ from that of the seawater to which the animals were acclimated. Moreover, these bloods also lacked absorption maxima in the region of 345 nm, as did plasma of the RBC-containing pteriomorph *Noetia ponderosa* (C. P. Mangum, unpub. obs).

The higher bivalve taxa, including the Heterodonta (which contains most of the familiar species), are regarded as suspect (Newell, 1969). But the Pteriomorpha, a relatively primitive group consisting of the anisomyarians, the extinct cyrtodonts, and the RBC-containing arcoids, appears to be a natural taxon. In addition, there is general agreement that the protobranch bivalves are even more primitive than the pteriomorphs (Newell, 1969; Allen, 1978, 1986). This relationship has several implications for the question of the origin and further evolution of O₂ transport systems, because it is among the pteriomorphs that one finds RBCs resembling counterparts and containing Hbs similar to those in other phyla at comparable levels of organization. The subject is of such importance that it is discussed in detail below.

Nucleated RBCs containing either monomeric or oligomeric Hbs are found in seven animal phyla (summarized by Mangum and Mauro, 1985), including five (Phoronida, Annelida, Echiura, Nemertina, and Mollusca) that are often regarded as not too distantly related to one another and at an intermediate stage of phylogenetic development (Fig. 7A). While the limited anatomical information indicates the possibility of some distinctly different features of the RBCs in each group, it also indicates many similarities. The physiological information, also limited, indicates a similar metabolic organization of at least annelid and molluscan RBCs, which differs from that of avian and mammalian RBCs and, possibly, the sipunculid pink blood cell (Mauro and Isaacks, 1984; Mangum and Mauro, 1985).

Within the molluscs, RBCs occur widely in one order of pteriomorph bivalves (as well as in a single species of heterodonts; Terwilliger *et al.*, 1983), and they almost certainly occur in the Aplacophora, which was regarded by Hyman (1967; p. 69) as the "genuinely primitive" molluscan class (Fig. 7B). Hyman (1967; p. 65) noted that "the coelomic fluid has a reddish hue invested in the corpuscles, except in the Chaetodermidae, where the red substance, not proved to be hemoglobin, is dissolved in the fluid itself." Despite the caveat, it is highly likely that this pattern reflects yet another instance of O₂ transport by RBCs (see also Baba, 1940) as the primitive condition and of multidomain, extracellular Hb as the derived condition. Well known examples include the annelids as well as the lamellibranch bivalves. RBCs containing simple heme proteins are found in more primitive species and extracellular Hbs that differ fundamentally from one another as well as from the simple heme proteins occur in more advanced taxa. One pteriomorph bivalve, believed to represent the transitional stage, has both kinds of Hbs in its RBCs (Grinich and Terwilliger, 1980).

There is a strong possibility that the nemertines exhibit the same trend. Hyman (1951; p. 490) believed that the red color of the blood "resides in the corpuscles," which is true of a few marine species (Vernet, 1979). In support of this contention Hyman (1951) cited the 1872 report by Lankester, whose words indicate otherwise: "the colour is due to Haemoglobin *diffused* in the liquid" (p. 73, italics ours). Poluhovich (1970; also pers. comm.), who reported Hb in freshwater (and therefore not primitive) nemertines, did not detect RBCs. Outside of the vertebrates, RBCs are unknown in freshwater animals, which is believed to be due to their osmotic fragility (Mangum, 1980a).

On the basis of the distribution of the RBC summarized above and illustrated in Figure 7, there is no compelling reason to postulate more than one origin of the RBC and its simple hemoglobins. One need only to suppose that the RBC originated

shortly after circulating body fluids arose (Fig. 7). In more advanced groups it was repeatedly replaced by extracellular O_2 carriers due to the greater viscosity of the large bore tubes that dominate primitive cardiovascular systems (Mangum, 1976), and it was inherited by two more advanced deuterostome groups: the echinoderms and the chordates.

The most obvious interpretation of the existence of Hc in the blood of proto-branch bivalves is that the hypothesis of a common origin of the RBC is incorrect. This interpretation has the following implications: Protobranchs represent the ancestral bivalve condition and their HcO_2 transport system was either lost (anisomyarians) or replaced (arcoids) in the pteriomorphs by an intracellular HbO_2 transport system of *de novo* origin. This interpretation is consistent with the presence of neuro- and branchioglobin in the same individuals that contain Hc. It is also consistent with the recent finding that the tertiary structures of the simple Hbs found in the annelids, bivalves, and primitive vertebrates are similar to one another and to mammalian myoglobin, and different from that of higher vertebrates Hbs (Perutz, 1985; Royer *et al.*, 1985; W. E. Love, pers. comm.). According to this multiple origin hypothesis, structural similarity of the simple Hbs of the lower animals is due to two separate origins from their tissue heme proteins, which also have the same tertiary structure (presently unknown, but probable), not a common origin.

The weaknesses of this interpretation include uncertainty about the integrity of the class Bivalvia (McAlester, 1966; Cox *et al.*, 1969; Newell, 1969; Runnegar, 1978) and the absence of a clear selection pressure for the replacement of Hc with RBCs. The simple heme proteins found in pteriomorph RBCs would seem to offer no clear advantages over protobranch Hcs. Indeed, their respiratory properties are not nearly as plastic, at least under physiological conditions (Mangum, 1980a), and therefore the selection pressure would seem to be negative.

Two alternative interpretations seem so unlikely that they can be dismissed with some confidence. (1) The hypothesis that the RBC and its simple Hbs had a common origin and that protobranch RBCs were lost in favor of a HcO_2 transport system after the divergence of the protobranchs and the pteriomorphs entails at least two independent origins of molluscan Hc culminating in similar quaternary structures with very different functional properties. As pointed out in detail earlier (Mangum *et al.*, 1985), this absence of correlation between known aspects of quaternary structure and respiratory function fails to provide a selection pressure for convergent evolution of those aspects of structure and it implies that they are conservative, ancestral characters. The point is strengthened by the present finding of a similar quaternary structure of bivalve and other molluscan Hcs, with a strikingly different combination of respiratory properties. (2) The notion that the RBC had a common origin and that the pteriomorphs, not the protobranchs, are the more primitive bivalves can also be dismissed. Abundant morphological evidence indicates otherwise.

A third alternative, the hypothesis that the RBC had a common origin and that the Protobranchia and the Pteriomorpha did not have a common ancestor, is somewhat more difficult to reject. While the possibility of a di- and even polyphyletic origin of the bivalves is frequently mentioned (*e.g.*, Cox *et al.*, 1969; Newell, 1969; Runnegar, 1978), the position has been advocated positively and forcefully only with respect to a separate origin of the Lucinacea (McAlester, 1966), not separate origins of the Protobranchia and at least one other lineage containing the Pteriomorpha and the (infrequently) Hb-containing Heterodonta. The strongest supporting evidence may be the results reported by Purchon (1978), who employed a matrix analysis based on set theory to cluster and thus to gauge the degree of relatedness between the 40 recent bivalve superfamilies whose taxonomic integrity is uncontroversial. Using the

nine (of 17) characters that Newell (1969) had designated as diagnostic of the superfamilies and that were either practical or suitable for the analysis, Purchon (1978) identified only two major clusters of bivalves, the nuculoids and the rest. His conclusion is reflected in Allen's (1986) diagnosis of the two quite different bivalve subclasses. With the stipulation that the nuculoid cluster should include the solemyoids, as in Allen's (1986) scheme, the present findings identify a tenth character that supports the notion of one and only one major "taxochasm" among the bivalves (Purchon, 1978). We mention the possibility of diphyly less in advocacy of it than as an alternative that circumvents the weaknesses of the hypothesis of multiple origins of the RBC and which, therefore, should not be ignored.

Further progress in understanding the evolution of O₂ transport systems awaits further elucidation of the structure and function of tissue heme proteins and also further understanding of how bivalve O₂ transport systems work. When details such as blood gas levels, pH, responses to hypoxia, respiratory and cardiovascular design constraints, etc. are known in protobranchs as well as additional Hb-containing bivalves, then the selection pressures favoring the evolution of systems with particular properties will become clearer.

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PARTICLE SIZE AND FLOW VELOCITY INDUCE AN INFERRED SWITCH IN BRYOZOAN SUSPENSION-FEEDING BEHAVIOR

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ABSTRACT

The feeding rates of two bryozoan species varied with particle size and flow velocity. In one species, increased flow reduced feeding on larger particles. The anomalously high capture rate of the largest particles by the smaller of the two species indicates a switch in feeding by ciliary currents to feeding that involves a high degree of tentacular activity. This is the first quantification of feeding under alternate modes in a benthic invertebrate and suggests that tentacular feeding may provide a significant source of nutrition for bryozoans.

INTRODUCTION

It is increasingly clear that switches in feeding strategies are common in benthic marine organisms. Some polychaetes, clams, and amphipods switch from deposit to suspension feeding with increases in flow or suspended material; some corals both entrap zooplankters with their tentacles and use mucus to entangle suspended particles; and some active suspension feeders feed passively under certain conditions (*e.g.*, barnacles, ascidians, brachiopods, and sponges) (Lewis and Price, 1975; Taghon *et al.*, 1980; Dauer *et al.*, 1981; LaBarbera, 1984; Olafsson, 1986). There is also evidence for alternate feeding modes in zooplanktonic suspension feeders. Copepods have been argued to filter feed on small particles and to actively grasp particles of larger size (*e.g.*, Conover, 1966; Richman and Rogers, 1969; Poulet, 1974) [but see more recent clarification of copepod feeding by Koehl and Strickler (1981)]. In addition, many suspension feeders take up dissolved organic matter, although the process is presumably continuous and would entail no switch in feeding behavior (Stephens and Schinske, 1961; Jørgensen, 1976; DeBurgh and Fankboner, 1978; Stewart, 1979).

In bryozoans, feeding currents produced by cilia lining the tentacles of the lophophore can be accompanied by a high degree of tentacular activity ranging from simple individual tentacle-flicking to encaging particles with all of the tentacles (Winston, 1978). Encagement activity is generally observed when particles are large; however, there has been no explicit test of the factors promoting greater tentacular *versus* ciliary feeding in bryozoans. In addition, the amount of food ingested under alternate modes has not been determined for any benthic invertebrate. This study compares the effects of particle size and ambient flow velocity on the feeding of the two closely related arborescent bryozoans, *Bugula neritina* and *B. stolonifera*.

Bryozoans are exclusively colonial animals common in both modern and fossil marine habitats. A variety of colony morphologies are found among bryozoan spe-

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TABLE I

Dimensions¹ of Bugula neritina and B. stolonifera

	<i>Bugula neritina</i>	<i>Bugula stolonifera</i>
Maximum size of colony (cm)	8	3-4
Number of tentacles	23-24	13-14
Mean length of tentacles (cm)	6.16×10^{-2}	4.47×10^{-2}
Mean diameter of lophophore (cm)	7.64×10^{-2}	4.41×10^{-2}
Mean diameter of mouth (cm)	7.4×10^{-3}	4.9×10^{-3}

¹ Values from Ryland and Hayward (1977) and Winston (1978, 1982).

cies. These include encrusting, arborescent, and massive colonies. The zooids (modules) that compose bryozoan colonies also vary substantially in form and function both among species and within colonies of the same species. Feeding zooids ingest suspended particles using the ciliated crown of tentacles, the lophophore. Gut contents and laboratory rearing experiments indicate that flagellates and diatoms can be important food items (Winston, 1977, and references therein). However, little is known of the food sources for field populations since plankton is inherently patchy in nature, the partial digestion of gut contents hinders identification of ingested material, and the small size of feeding zooids and the even smaller size of their prey make direct observation in the field difficult. The effect of flow on bryozoan feeding has received some attention (Okamura, 1984, 1985), but the effect of particle size is unknown.

MATERIALS AND METHODS

Colonies of the arborescent, anascan cheilostomes *Bugula stolonifera* and *B. neritina* co-occur in fouling communities that develop on submerged structures in ports and harbors. In Florida, colonies occur on seagrasses, oyster shells, docks, canal walls, floats, rotting wood, algae, coastal rock ledges, and inlet breakwaters (Winston, 1982). Both species are widespread. *B. stolonifera* is the smaller species (see Table I) and often colonizes and grows within *B. neritina* colonies. In this study, *B. neritina* and *B. stolonifera* colonies were collected from the floating docks of the Harbor Branch Oceanographic Institution at Link Port, Florida. Laboratory observations of bryozoan feeding behavior confirm that high degrees of tentacular activity and encagement of particles occur in several *Bugula* species (including *B. neritina*) and seem to be associated with particle size and motility (Winston, 1978). However, these observations were made in still water.

Feeding experiments were performed by submerging colonies in a recirculating flow tank (Vogel and LaBarbera, 1978) in which currents of known mean velocities could be created in the working section (13 cm × 13 cm × 13 cm). The flow tank contained a suspension of latex particles (polystyrene divinylbenzene calibration particles: Duke Scientific Corporation, Palo Alto, CA). (Initial observations showed that the bryozoans would ingest these particles.) Two flow velocities were created in the flow tank: a relatively slow flow (1-2 cm/s) and a relatively fast flow (10-12 cm/s). Flow measurements taken with an electromagnetic flow probe (Marsh McBirney No. 523) in the field at the branch tip level of *Bugula stolonifera* (Okamura, 1984) indicate that both species encounter flow velocities in the experimental range (measurements

were made in a habitat where both species occurred). Feeding on three sizes of latex particles was assessed at each flow velocity. Particle diameters were 9.6 (SD = 0.5), 14.6 (SD = 1.0), and 19.1 (SD = 1.1) microns. At the outset of experiments, particle concentrations in the flow tank were set at 100 particles/ml by adding appropriate volumes of stock suspensions of known concentrations to a known volume of filtered seawater in the flow tank. Concentrations of 100 particles/ml lie well within the range of concentrations of flagellates measured in the field (*e.g.*, Jørgensen, 1966; Bullivant, 1968). Control runs in the flow tank indicated that latex particles do not settle out of suspension over time at either flow velocity employed (Okamura, 1984).

Up to three replicate colonies were placed in the flow tank during a given experiment. Only portions of colonies were used in all experiments. [Clipping colonies does not affect feeding activity (Okamura, 1984)]. Colonies were allowed to feed for 20 min and then were removed from the flow tank and placed in dilute sodium hypochlorite. This treatment dissolves the organic contents of colonies but leaves intact the exoskeleton, membranous material, and latex particles. Ingested latex particles (that can initially be discerned only poorly in the gut before the gut wall dissolves) remain trapped within the zooidal exoskeleton and membranes and can be counted easily. These counts provided an estimate of the mean number of particles ingested per feeding zooid per colony (range of zooids sampled per colony = 5–100, mean = 64.4, SD = 35.2) (range of colonies replicated per treatment = 8–16). The effects of flow velocity and particle size on the mean number of particles ingested per zooid per colony were then analyzed with two-way analyses of variance for each species.

RESULTS

Bugula neritina ingested few small particles at both flow velocities (see Fig. 1A). More large particles were consumed than medium-sized particles in slow flow. This pattern reversed itself in fast flow (note the nearly significant interaction term). The smaller *B. stolonifera* showed greatest ingestion of medium-sized particles in slow flow (see Fig. 1B). Feeding on medium-sized and small particles was inhibited in fast flow; however, large particles were captured in great numbers.

DISCUSSION

Feeding patterns and their causal explanations

Rubenstein and Koehl (1977) used aerosol models of filtration to clarify suspension-feeding mechanisms, however these models can only be applied to passive suspension feeders. Because bryozoans and other active suspension feeders generate feeding currents, complex three-dimensional flow patterns arise between self-generated feeding currents and local currents near the feeding structures (Jørgensen, 1980). For feeding to occur, particles must be transferred from local currents into the self-generated feeding currents, and in doing so they must pass through a boundary zone characterized by steep velocity gradients (Jørgensen, 1980). The behavior of particles that enter steep velocity gradients is uncertain (Strathmann, 1982). With this in mind, several factors may explain the patterns of feeding from different flows on particles of varying size by *Bugula stolonifera* and *B. neritina*.

Large-sized particles travel with greater momentum and thus may be carried further downstream before crossing flow lines in velocity gradients. Strathmann (1971, 1982) argued that no evidence indicates that momentum carries particles across flow lines so that they will impinge upon the ciliary tracts of echinoderm larvae and lopho-

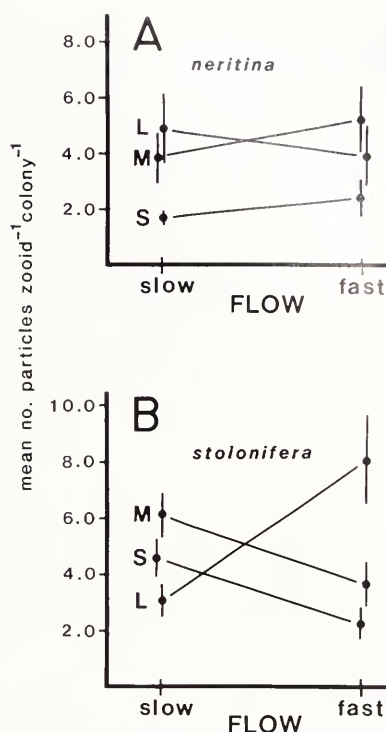


FIGURE 1. Mean number of 9.6 (S), 14.6 (M), and 19.1 (L) micron particles captured per feeding zooid per colony (± 2 SE) by *Bugula neritina* (A) and *B. stolonifera* (B) in slow and fast flow. Two-way analysis of variance for *B. neritina*: $F_{1,55}$ (velocity term) = 1.347, $P = 0.251$; $F_{2,55}$ (particle size term) = 18.120, $P < 0.001$; $F_{2,55}$ (velocity $\times$ particle size interaction) = 2.931, $P = 0.062$. Two-way analysis of variance for *B. stolonifera*: $F_{1,59}$ (velocity term) = 0.035, $P = 0.0851$; $F_{2,59}$ (particle size term) = 11.183, $P < 0.001$; $F_{2,59}$ (velocity $\times$ particle size interaction) = 42.679, $P < 0.001$.

phorates, and hence that momentum does not play a role in the suspension feeding of these organisms. However, the role of momentum in the transport of particles out of local currents and into feeding currents is unknown. Alternatively, the relatively greater drag experienced by large-sized particles may act to sweep them further downstream before crossing flow lines in velocity gradients. Both momentum and drag increase with ambient flow velocity.

The larger lophophores of *Bugula neritina* create stronger feeding currents, and these may account for its greater effectiveness in capturing large particles from slow flow. For *B. stolonifera*, highest ingestion rates in slow flow were on medium-sized particles. In fast flow, *B. neritina* was hindered in feeding on large particles and showed highest ingestion rates on intermediate-sized particles. The greater momentum of or drag on large particles in faster flow may make their ingestion more difficult. However, anomalously high feeding rates on large particles in fast flow were observed for *B. stolonifera*. The most likely explanation for this is a switch in feeding from mainly ciliary currents to feeding that involves a high degree of tentacular activity. Unfortunately, a switch in feeding technique was not anticipated and, consequently, lophophore behavior was not observed with a microscope during the experiments.

Furthermore, colonies were fixed and sampled when time permitted. When the feeding patterns were eventually discerned, there were no colonies available for observing lophophoral behavior (both species are highly seasonal in Florida). However, as mentioned earlier, laboratory observations indicate that feeding in many *Bugula* species does involve high degrees of tentacular activity and engagement (Winston, 1978). Only moderate levels of tentacular activity were observed in *B. stolonifera*, but since Winston's observations were made in still water and the sizes of suspended particles were unspecified, conditions that would have invoked high degrees of tentacular activity or engagement by *B. stolonifera* may not have been present.

The inferred switch in feeding mode by *Bugula stolonifera* is induced by increased flow rate and depends on particle size. The larger *B. neritina* showed no evidence of a switch in feeding behavior. However, if particles of larger size or perhaps if faster flow velocities had been employed, a switch in feeding would be expected. High degrees of tentacular activity were observed for *B. neritina*, including the formation of cages with its tentacles (Winston, 1978). The reduction in feeding on small and medium-sized particles with increased flow by *B. stolonifera* is in accord with previous results (Okamura, 1985).

Best and Thorpe (1983, 1986) provide evidence that bryozoans are capable of altering the strength of their feeding currents and do so in response to particle concentration. An alternate explanation for the present results is that the feeding patterns are produced by feeding currents of different strengths. If this is so, the greater flux of large particles in fast flow would induce *Bugula stolonifera*, but not *B. neritina*, to produce stronger feeding currents. While this is a possibility, it is considered unlikely since *B. stolonifera* fed disproportionately on large particles present in mixtures (composed of equal proportions of all three particle sizes) in fast flow (Okamura, in prep). In this case, if stronger feeding currents were produced, particles of all three sizes would be expected to be ingested in equal proportions. It is more likely that the disproportionate ingestion of large particles from mixtures was a result of selective tentacular feeding.

It is notable that the apparent switch in feeding by *Bugula stolonifera* entails such a large increase in capture. Tentacular feeding may involve a much greater energetic expenditure than ciliary feeding. Only when the gain is great (*i.e.*, many large particles per unit time) will feeding that involves a high degree of tentacular activity be a worthwhile strategy. Note that a switch to tentacular feeding results in a much greater amount of "biomass" captured [mean mass of large particles captured by *B. stolonifera* in fast flow = 30.80×10^{-9} g (SD = 8.50), of medium-sized particles = 6.21×10^{-9} g (SD = 1.87), and of small particles = 1.09×10^{-9} g (SD = 0.42)]. However, the costs of ciliary and tentacular feeding are unknown. The reduced surface area offered by small particles for tentacular contact may preclude tentacular feeding on particles below a minimum size, or perhaps particles must exceed a certain relative size to be perceived individually.

Plasticity in feeding behavior and its implications

It is evident that many suspension feeders are capable of great plasticity in feeding behavior. Alternate feeding techniques are invoked by variations in the suspension from which they capture their food. These variations may be characteristics of the prey items (*e.g.*, size, motility, chemistry) or physical properties of the medium itself (*e.g.*, temperature, density, and the patterns of fluid flow). Since suspension feeders will regularly encounter suspensions that vary in both physical properties and prey items, plasticity in feeding response is expected. The study of suspension feeding in

still water on uniform particles may often provide an incomplete picture of the feeding of many organisms. This is exemplified by the studies of Best and Thorpe (1983, 1986). They argue that tentacular flicking and the more localized ciliary reversal mechanism that Strathmann (1982) proposes to account for particle capture during ciliary feeding are not the main methods of feeding employed by bryozoans. They suggest that, overall, the bulk of particles ingested are those that feeding currents carry down the center of the lophophore towards the mouth. The importance of ciliary reversal and tentacular activity in feeding are rejected on the basis of calculating the number of reversals and tentacular flicks required to explain the ingestion rates they observed. However, their evidence may be biased due to their use of extremely high particle concentrations (50–200 cells μl^{-1}), small particle sizes, and the absence of ambient currents in their experiments. Their study suggests that very high particle concentrations may swamp contributions to feeding by mechanisms other than the bulk flow of particles through the center of the lophophore, while this study suggests that high degrees of tentacular activity depend on both particle size and ambient flow.

Many investigators have studied the relationship between bryozoan colony form and the patterns of self-generated feeding currents through colonies (Cowen and Rider, 1972; McKinney, 1977, 1986a, b; Taylor, 1979; Anstey, 1981; McKinney *et al.*, 1986). Results reported here indicate that feeding currents may not always be of primary importance in particle capture. The potential for alternate feeding behaviors should be appreciated when interpreting colony morphology solely in terms of feeding current patterns. Both stenolaemate and gymmolaemate bryozoans display high degrees of tentacular activity even in still water (Winston, 1978). Tentacular feeding from faster ambient flow and/or on certain types of prey may provide a significant source of nutrition for a variety of bryozoans.

Optimal foraging theory attempts to explain and predict many aspects of the foraging behavior of animals by assessing foraging tactics in terms of maximizing net rates of energy gain and therefore fitness (*e.g.*, Schoener, 1971; Pyke 1977, 1984; Hughes, 1980). Which prey will be the “best” is determined by the energy content of the prey and the energetic cost to the predator of searching for and handling the prey. Thus, understanding patterns of prey selection, prey vulnerability, and feeding behaviors is crucial in the interpretation of foraging strategies. Particle size appears to relate to prey vulnerability in bryozoan feeding. Flow velocity induces a switch in feeding behavior that results in a shift in the size of particles captured. Furthermore, flow velocity appears to control the vulnerability of particles of certain size ranges even when feeding under one mode (note greater feeding on large particles in slow flow but on medium-sized particles in fast flow by *Bugula neritina*). Prey vulnerability and patterns of prey capture are thus determined by both the constraints imposed by flow and by the flow-induced change in feeding tactics. This suggests that the role of flow on particle size selection and the behavior of suspension feeders merits further investigation. In addition, a switch in feeding tactics by bryozoans implies that these organisms perceive and assess prey availability and subsequently adopt the most efficient feeding mode (*i.e.*, the one that maximizes net energy gain). It appears that predictions of optimal foraging theory may be applicable to benthic suspension feeders despite their seemingly simple sensory capabilities and sessile existence.

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EPITHELIAL WATER PERMEABILITY IN THE EURYHALINE MUSSEL *GEUKENSIA DEMISSA*: DECREASE IN RESPONSE TO HYPOOSMOTIC MEDIA AND HORMONAL MODULATION¹

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ABSTRACT

The diffusional water permeability of isolated mantles from the mussel *Geukensia demissa* was reduced by incubation of the tissues in hypoosmotic media. The permeability of mantles from 1000 mOsm seawater (SW)-acclimated animals was 6×10^{-5} cm/s. A four-hour incubation in 500 mOsm SW or 250 mOsm SW reduced the water permeability by 2×10^{-5} cm/s and 4×10^{-5} cm/s, respectively. A half-hour exposure to the hypoosmotic medium was sufficient to induce the decrease in permeability.

The water permeability of mantles incubated in isosmotic SW containing acetone extracts of ganglia from 1000 mOsm SW-acclimated mussels or of mantle from 500 mOsm SW-acclimated mussels was significantly reduced. Extracts of gill had no effect.

Ovine prolactin (50 mg/ml) decreased the water permeability of mantles in isosmotic seawater. Cortisol (10^{-4} M), arginine vasopressin (10^{-6} M), and the molluscan neuropeptide FMRFamide (10^{-6} M) had no effect.

These results show that the epithelial water permeability of euryhaline bivalves varies with changes in the ambient salinity, and that these permeability changes may be modulated by factors of neural origin.

INTRODUCTION

A number of specific physiological mechanisms facilitate the survival of euryhaline marine animals in habitats characterized by variations in salinity. These mechanisms include changes in urinary output, regulation of the extracellular fluid composition, volume regulation, and changes in epithelial permeability to water and ions.

A reduced epithelial permeability to water in dilute media has been reported in several invertebrates, including a number of arthropods (Rudy, 1967; Smith, 1970a; Capen, 1972; Smith and Rudy, 1972; Cornell, 1973; Hannen and Evans, 1973; Lockwood *et al.*, 1973; Rosejidi *et al.*, 1976; Thuet, 1978), three polychaetes (Smith, 1964, 1970b; Fletcher, 1974), and a bivalve (Prusch and Hall, 1978). The phenomenon has been observed in osmoconformers (*e.g.*, *Mytilus edulis*, *Libinia emarginata*) and in osmoregulators (*e.g.*, *Rhithropanopeus harrisi*, *Nereis limnicola*). Nearly all of these data were collected by measuring changes in the fluxes of water into or out of whole animals exposed to dilute media. As indicated by Cornell (1979), part of the observed decreases in the flux of water across the epithelium of an intact animal could be effected by changes in circulation or ventilation. This criticism does not apply to

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studies of the response of the epithelial permeability of isolated tissues to changes in salinity.

Isolated tissues from only a few marine invertebrates have been used to examine changes in water permeability in response to dilution of the medium. Cantelmo (1977) found that the water permeability of gut epithelia and gills from the crabs *Cancer irroratus* and *Callinectes sapidus* was lower in tissues isolated from animals acclimated to 40% seawater than in tissues from animals acclimated to 100% seawater. However, exposure of isolated tissues to hypoosmotic media for 2 h did not change their water permeability. Acclimation of the mussel *Mytilus edulis* to reduced salinity caused a decrease in the diffusional permeability of water across isolated mantle tissues (Prusch and Hall, 1978).

Neurohormones have been implicated in the modulation of water permeability in a variety of invertebrates. For example, extracts of the thoracic ganglion decrease the water flux across isolated crab gut and gills (Mantel, 1968; Berlind and Kamemoto, 1977), and the injection of a brain homogenate reduces the rate of water exchange of earthworms (Carely, 1981). Two lines of evidence suggest a possible modulation of water balance by neural products in gastropod molluscs. Injection of synthetic thyrotropin releasing hormone into the freshwater snail *Lymnaea stagnalis* causes a slight loss in wet weight (Grimm-Jorgenson, 1979). Similarly, injection of homogenized R-15 cells from the abdominal ganglion of the opisthobranch *Aplysia brasiliensis* resulted in a 5% gain in wet weight (Kupfermann and Weiss, 1976).

The present study was undertaken to determine the response of the epithelial water permeability of isolated mantle tissue from the euryhaline mussel *Geukensia demissa* to decreases in the ambient osmotic concentration. This tissue was also used as a bioassay system to determine the effects of extracts of the ganglia and other tissues on water permeability.

The results show that the diffusional water permeability (P_d) of the mussel decreases in response to the dilution of the external medium, and that water permeability may be modulated by a factor of neural origin.

MATERIALS AND METHODS

Animals

Atlantic ribbed mussels, *Geukensia demissa granosissima*, were collected from a salt marsh near St. Augustine Beach, Florida. The animals were kept unfed in running seawater (30‰) at ambient temperature. All animals were used within 3 weeks of collection.

Histology

The adductor muscles of individual mussels were cut, the animals opened, and pieces of the central portion of the mantle dissected free. The tissue was fixed in filtered seawater containing 2% glutaraldehyde, dehydrated and cleared in a graded series of water/ethanol/t-butyl alcohol, and embedded in paraffin. Sections (10 μm) were cut on a microtome, stained with hematoxylin eosin, and mounted on glass slides.

Measurement of diffusional water permeability

The adductor muscles of individual mussels were cut and the valves carefully pried apart. Each mussel provided two tissues, the mantle covering the inside of the

right valve and the mantle covering the inside of the left valve. The left and right mantle halves were cut away from the visceral mass and detached from the margins of their respective valves. The isolated tissues were placed in small covered dishes containing 5 ml of 1000 mOsm seawater (SW) which was aerated via small bore tubing. After 60 minutes of incubation, one mantle half was mounted over the aperture of one half of a diffusion chamber and secured with a soft rubber o-ring. The tissues were always mounted in the chamber so that the movement of tritiated water was from the extrapallial cavity side to the mantle cavity side. Five ml of medium were placed in the chamber to provide a hydrostatic pressure head sufficient to check the mounted tissue for obvious leaks. The chamber was then assembled and both sides filled with medium by alternate additions of 2–3 mls. The total volume of each compartment of the chamber was 14 ml; the area of exposed tissue was 1 cm². Mixing and aeration were provided by gas lift pumps powered by water-saturated air. About 1 μ Ci of tritiated water was added to one compartment of the chamber (the "hot" side), and following 10 minutes equilibration, 100 μ l samples of the medium in the other compartment (cold side) were removed at 15 minute intervals for 60–90 minutes. These samples were mixed with 10 ml scintillation cocktail and counted on a liquid scintillation counter. In experiments using paired left and right mantles, the flux across one mantle was measured while the matching tissue was transferred to a dish containing either control (1000 mOsm SW) or experimental medium. These matching tissues were further incubated from one half to four hours and then their P_d values determined in the same rinsed and dried chamber used for the matching control measurement.

The water flux across the tissue and the diffusional water permeability were calculated from equations 1 and 2, respectively:

$$1. \quad J_{12}^{H_2O} = \left(\frac{1}{t} \right) \frac{Q_2^*}{Q_1^*/Q_1} \left(\frac{1}{A} \right) \quad \begin{array}{l} Q_1^*/Q_1 = \text{specific activity in compartment 1} \\ Q_2^* = \text{amount of isotope in compartment 2} \\ t = \text{time} \\ A = \text{area of tissue} \end{array}$$

$$2. \quad P_d = \frac{J_{12}^{H_2O}}{C_w} \quad C_w = \text{molar concentration of water}$$

The differences in P_d values between paired left and right mantles were averaged and differences among treatment means assessed by Student's *t* test.

Tissue extracts

The pedal, visceral, and cerebral ganglia from 250 mussels acclimated to 1000 mOsm SW were dissected from the animals and pooled in a large volume of cold acetone to extract putative hormones and inactivate proteolytic enzymes; acetone extracts were also made of the gills and mantles from these mussels. The mantles of 200 mussels acclimated to 500 mOsm SW were also extracted in acetone. The extracts were evaporated to dryness on a rotary evaporator and the water soluble portion of the residue taken up in a minimal volume of distilled water. The dose added to the incubation media was approximately 1 animal equivalent. All tissue extracts and hormones were added to the incubation media and to the fluid in both compartments of the diffusion chamber.

RESULTS

Histology

Figure 1 shows a cross section of the central portion of the mantle. This complex tissue separates the extrapallial space from the mantle cavity. Both surfaces are lined

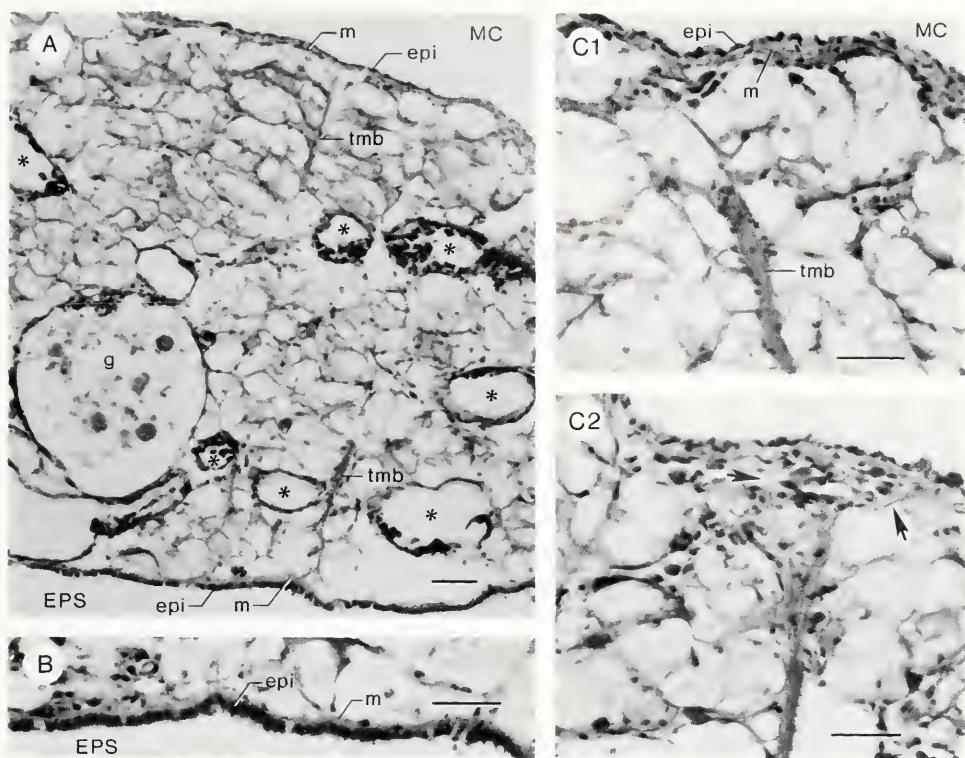


FIGURE 1. Cross-section through the central portion of the mantle of *Geukensia demissa*. A. Section from the mantle cavity (MC) to the extrapallial space (EPS) showing the epithelia (epi) on both surfaces with underlying muscle layers (m). The epithelia are separated by connective tissue which encompasses many hemolymph vessels (*), few genital follicles or canals (g), and transverse muscle bundles (tmb) connecting the two subepithelial muscle layers. Bar = 200 μ m. B. Columnar extrapallial epithelium with thin underlying muscle layer. Bar = 100 μ m. C. Squamous mantle cavity epithelium with thick underlying muscle layer. Fibers from the transverse muscle bundles splay out to join the subepithelial muscle layer (arrows in C2). Bar = 100 μ m.

with an epithelium underlain by a muscle layer (Fig. 1A). The extrapallial epithelial cells are much taller than those lining the mantle cavity, but the subepithelial muscle layer associated with the extrapallial space is much thinner than that on the mantle cavity side of the tissue (compare Figs. 1A, 1C₁). The bulk of the mantle is occupied by connective tissue in which are found numerous hemolymph vessels and, in these non-reproductive specimens, occasional genital canals (Fig. 1A). Bundles of muscle fibers traverse the mantle joining the two subepithelial muscle layers (Figs. 1A, 1C₂). Similar structures have been described for the mantles of other species (Beedham, 1958).

Diffusional water permeability

Preliminary experiments showed that the accumulation of counts in the cold compartment of the diffusion chamber was linear with time for over six hours, indicating that the 10 minute equilibration with labelled water was sufficient for attainment of a steady-state flux across the tissue. The diffusional water permeabilities of

TABLE I

*Changes in diffusional water permeability (P_d) of mantles from 1000 mOsm seawater-acclimated *Geukensia demissa* after a four hour incubation in various seawaters*

Treatment medium	1000 mOsm	500 mOsm	250 mOsm
Change in P_d	0.5×10^{-5}	*** -2.3×10^{-5}	*** -4.1×10^{-5}
SD	1.1×10^{-5}	3.0×10^{-5}	1.3×10^{-5}
n	18	9	7

Values are in cm/s and represent differences in P_d between paired left and right mantles: one mantle of each pair was incubated in 1000 mOsm SW for 1 h; the other was incubated in the treatment medium for 4 h. Values significantly different from the 1000 mOsm treatment are marked with *** ($P < .001$).

mantles from animals acclimated to 1000 and 500 mOsm for three weeks were, respectively, $7.9 \pm 3.3 \times 10^{-5}$ ($n = 10$) and $4.3 \pm 0.7 \times 10^{-5}$ cm/s). These values are higher than the mean (2.2×10^{-5} cm/s obtained by Prusch and Hall (1978) for mantles of *G. demissa* acclimated to 1000 mOsm SW. Their animals, collected near Woods Hole, Massachusetts, were undoubtedly the subspecies *G. demissa demissa*. Differences in chamber design and differences between the two *G. demissa* subspecies probably account for the discrepancy.

There were no significant differences between the mean P_d values of mantles incubated for four hours in 1000 mOsm SW ($6.1 \pm 2.9 \times 10^{-5}$ cm/s) and the mean P_d s of the matching control (incubated in 1000 mOsm SW for 1 h) tissues ($6.5 \pm 2.9 \times 10^{-5}$ cm/s), nor was the mean of the differences between paired tissues ($0.5 \pm 1.1 \times 10^{-5}$ cm/s) significantly different from zero. In contrast, the water permeability of mantles incubated in 500 or 250 mOsm SW for four hours was decreased by $\frac{1}{3}$ and $\frac{2}{3}$, respectively, compared to paired controls (Table I). The data from a representative experiment are shown in Figure 2. The movement of labelled water across both tissues is linear with time; the movement of water across the tissue incubated in 500 mOsm SW is slower. The magnitude of the reduction in flux is constant throughout the experiment.

The time course of the reduction of epithelial water permeability (P_d) in 500 mOsm SW is summarized in Table II. A thirty minute incubation in dilute seawater was sufficient to induce a decrease of about 1×10^{-5} cm/s in the P_d value. Longer incubations further reduce the permeability, but these values were not significantly different from that induced by a thirty minute incubation (Table II).

When mantles were incubated in isosmotic medium containing an extract of ganglia from 1000 mOsm SW-acclimated mussels or an extract of mantles from 500 mOsm SW-acclimated mussels, the water permeabilities were significantly reduced. Extracts of other tissues had no effect (Table III).

Hormones which affect the water permeability of vertebrate tissues were tested for effects on the bivalve mantle. Ovine prolactin significantly reduced the P_d value of mantle tissues in isosmotic media. Neither arginine vasopressin nor cortisol changed the permeability of mantles in isosmotic SW (Table IV). The small reduction in the P_d value of tissues incubated in isosmotic media with the molluscan neuropeptide FMRFamide was not significant (Table IV). Colchicine did not prevent the decrease in P_d induced by exposure to dilute media (Table V).

DISCUSSION

The diffusional water permeability (P_d) of isolated mantles from the euryhaline mussel *Geukensia demissa* decreases when the tissues are exposed to hypoosmotic

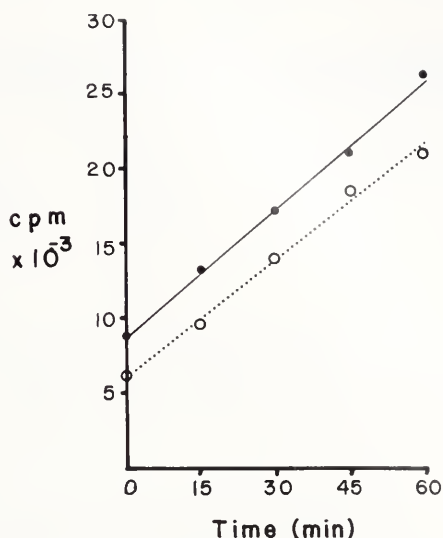


FIGURE 2. The unidirectional movement of tritiated water across a piece of isolated mantle of *Geukensia demissa*. Total counts per minute appearing in the "cold" side of a diffusion chamber are plotted as a function of time. The data are from paired mantle tissues from one mussel: the left mantle was incubated for 1 h in 1000 mOsm seawater (solid circles); the right mantle was incubated in 500 mOsm seawater (open circles) prior to measurement of the tritiated water flux in a diffusion chamber containing the same media.

media; the decrease in permeability is proportional to the magnitude of the decrease in the ambient osmotic concentration. Furthermore, the P_d of the isolated tissue incubated in isosmotic medium is reduced by a vertebrate hormone and by an endogenous factor of neural origin.

The reduction in permeability induced by a 30 min exposure to 500 mOsm seawater is less than that resulting from long-term acclimation of the mussels to 500 mOsm SW. The reduction of water permeability by ganglion extracts from 1000 mOsm-SW acclimated mussels and mantle extracts from 500 mOsm-SW acclimated mussels suggests that the putative factor is produced in the ganglia and released to the periph-

TABLE II

Time course of change in water permeability (P_d) of mantles from 1000 mOsm seawater-acclimated *Geukensia demissa* during incubation in 1000 mOsm or 500 mOsm seawater

	Treatment medium									
	1000 mOsm					500 mOsm				
Incubation duration (h)	0.5	1	2	3	4	0.5	1	2	3	4
P_d change ($\times 10^{-5}$ cm/s)	0.9	0.4	0.3	0.4	0.2	*-0.9	***-1.0	** -1.4	***-1.9	***-1.9
SD	1.1	0.4	1.4	0.9	0.9	0.6	0.9	1.4	1.8	1.4
n	4	3	4	8	5	4	10	9	10	7

Values are differences in P_d between paired left and right mantles: one mantle was incubated in 1000 mOsm SW for 1 h; the other was incubated in the treatment medium for 0.5 to 4 h. Values marked with asterisks are significantly different from the corresponding 1000 mOsm treatment value (* = $P < .05$, ** = $P < .01$, *** = $P < .001$)

TABLE III

The effect of various tissue extracts on the water permeability (P_d) of mantles from 1000 mOsm seawater acclimated Geukensia demissa incubated four hours in 1000 mOsm seawater

	1000 SW	Treatment medium			
		1000 SW + 1000 mantle ext.	1000 SW + 1000 gill ext.	1000 SW + 1000 ganglia ext.	1000 SW + 500 mantle ext.
P_d change	0.5	1.3	0.6	*-1.4	** -1.5
SD	1.4	1.1	1.6	1.2	1.9
n	18	6	5	3	10

Values are $\times 10^{-5}$ cm/s and represent differences in P_d between paired left and right mantles: one mantle was incubated in 1000 mOsm SW for 1 h; the other was incubated in the treatment medium for 4 h. Values marked by asterisks are significantly different from that for 1000 SW (* = $P < .05$; ** = $P < .01$).

ery during acclimation to low ambient salinity. Prusch and Hall (1978) observed a 67% reduction in the water permeability of mantles isolated from the mussel *Mytilus edulis* during four weeks of acclimation to 70‰ seawater. Thus, in the intact animal, continuous release of neural factors may facilitate a larger decline in permeability than occurs in isolated tissues. As yet there are no data on the size, structure, or chemical nature of this putative neurohormone. However, it is not FMRFamide (Table III).

Prolactin reduced the water permeability of *G. demissa* mantles in isosmotic media (Table IV). Prolactin also reduces the permeability of teleost epithelia to water and ions (Doneen and Bern, 1974; Foskett *et al.*, 1983). While the presence of prolactin has been demonstrated by immunocytochemical methods in ascidians (Pestarino, 1984), it has not been reported in any other invertebrate. Therefore it is unlikely that the active substance in *G. demissa* ganglia is prolactin.

Khan and Salueddin (1979; 1981) associated changes in the anatomy of the septate junctions between kidney cells in the snail *Helisoma duryi* with increased water permeability. Extracts of the visceral ganglia induce these changes which occur within

TABLE IV

The effects of selected hormones on the change in diffusional water permeability (P_d) of mantles from 1000 mOsm seawater-acclimated Geukensia demissa incubated four hours in 1000 mOsm seawater

	1000 SW	Treatment medium			
		1000 SW + Prolactin (50 μ g/ml)	1000 SW + FMRFamide (10^{-6} M)	1000 SW + cortisol (10^{-4} M)	1000 SW + Arg vasopressin (10^{-6} M)
P_d change	0.5	***-2.3	-0.4	-0.5	-0.4
SD	1.1	1.4	0.9	1.5	2.0
n	18	6	4	4	5

Values are $\times 10^{-5}$ cm/s and represent differences in P_d between paired left and right mantles: one mantle was incubated in 1000 mOsm SW for 1 h; the other was incubated in the treatment medium for 4 h. The value marked by *** is significantly different from that for 1000 SW ($P < .001$).

TABLE V

The effect of colchicine on the water permeability (P_d) of mantles from 1000 mOsm seawater-acclimated Geukensia demissa incubated four hours in 500 mOsm seawater

	Treatment medium	
	500 SW	500 SW + colchicine ($2 \times 10^{-4} M$)
P_d change	-2.3	-2.0
SD	3.0	3.1
n	9	3

Values are $\times 10^{-5}$ cm/s and represent differences in P_d between paired left and right mantles; one mantle was incubated in 1000 mOsm SW for 1 h; the other was incubated in the treatment medium for 4 h.

30 minutes. Neurohormones, then, can alter the water permeability of molluscan epithelia by causing changes in the structure of the tissues, thereby changing the resistance of the paracellular pathway to water movement. The failure of colchicine to prevent a decrease in the P_d of mantles exposed to dilute media (Table V) suggests that microfilament activity is not involved in the process.

If the major route of water movement across the *G. demissa* mantle is paracellular, osmotic swelling of the epithelial cells could contribute to a decrease in the water permeability of the tissue during exposure to hypoosmotic media. Isolated *G. demissa* ventricles exposed to hypoosmotic seawater stop beating, but the mechanical activity of the ventricle recovers within 90–120 minutes (Pierce and Greenberg, 1972). Recovery of the mechanical activity of the ventricle apparently is due to cellular volume regulation. If the time course of recovery of cellular volume by the mantle cells is similar to that of the myocardial cells, osmotic swelling cannot account for the reduction in water permeability induced by 2–4 h incubations in hypoosmotic seawater. However, in the absence of data on the time course of changes in the volume of mantle cells exposed to hypoosmotic stress, the possibility that cell swelling accounts for some or all of the decrease in epithelial permeability cannot be ruled out.

The mantle of *G. demissa* is vascularized (Fig. 1) and therefore well-perfused by the circulation. Mounting the tissues in the diffusion chamber precluded perfusion, and therefore the effects of delivery of the tissue extracts and other drugs via the circulation cannot be assessed.

Extracts of various nervous tissues affect epithelial water permeability of crustaceans and annelids (Mantel, 1968; Tullis and Kamemoto, 1974; Berlind and Kamemoto, 1977; Carely, 1981). While none of these factors has yet been identified, it is clear that neural factors modulate water permeability in euryhaline invertebrates.

In summary, the epithelial water permeability of euryhaline molluscs changes during acclimation to changes in the ambient salinity. These changes in permeability may be modulated by one or more neural factors of unknown structure. The mechanisms responsible for increases or decreases in water permeability apparently involve changes in the junctional complexes between the epithelial cells, but factors affecting transcellular water permeability, such as the insertion or removal of water channels or changes in the composition of the membrane lipid bilayer, cannot be ruled out.

ACKNOWLEDGMENTS

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METAL REGULATION AND MOLTING IN THE BLUE CRAB, *CALLINECTES SAPIDUS*: METALLOTHIONEIN FUNCTION IN METAL METABOLISM

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ABSTRACT

We recently demonstrated that zinc, copper, and hemocyanin metabolism in the blue crab varies as a function of the molt cycle. To extend these observations, and better delineate metal metabolism in marine crustaceans, we have conducted experiments to determine if environmental temperature and season of the year affect concentrations of hemocyanin and copper in the hemolymph and copper and zinc in the digestive gland. Overwintering, cold water crabs (6°C) had decreased hemocyanin and copper in the hemolymph and normal zinc and copper in the digestive gland with respect to summer crabs collected at 20–30°C. When these crabs were warmed to 20°C and fed fish for three weeks, they showed increases in the concentrations of copper in the digestive gland, and copper and hemocyanin in the hemolymph. In addition, a change from a zinc to a copper-dominated metallothionein was found in a majority of the warmed crabs, suggesting the involvement of copper metallothionein in the resynthesis of hemocyanin. Based on these observations and previous data (Engel, 1987) a conceptual model of copper and zinc partitioning in the blue crab has been constructed. In this model, metallothionein has an important role in metal regulation both during molting and in the changes related to season of the year. Metallothionein-bound copper and zinc appear to be regulated at the cellular level for the synthesis of metalloproteins, such as hemocyanin (copper) and carbonic anhydrase (zinc), both of which are necessary for normal growth and survival. Finally, we present evidence showing that copper metallothionein can directly transfer its metal to the active site of apohemocyanin. Copper insertion seems to precede the formation of viable oxygen binding sites.

INTRODUCTION

Studies of the reputed function of metallothionein in marine organisms have been concerned primarily with its role in detoxifying elevated concentrations of trace-metals accumulated from polluted environments (Roesijadi, 1981; Engel and Brouwer, 1984; and George and Viarengo, 1985). These reviews discussed the potential constitutive or regulatory function of metallothionein in metal metabolism, but emphasized its role in detoxifying metals.

Previously we alluded to the possibility that metallothioneins may play a role in organismal and cellular metal metabolism in marine species (Engel and Brouwer,

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1984; Engel and Roesijadi, 1987). Such suggestions also have been made concerning zinc and copper metabolism in mammals. Cousins (1982, 1985) discussed the role of metallothionein in zinc metabolism in rats. The observation that glucocorticoid hormones can significantly alter zinc metabolism and increase metallothionein synthesis in the liver without the administration of exogenous zinc also supports the hypothesis that metallothionein is active in normal metal metabolism (Karin, 1985). More recently Petering and Fowler (1986) discussed the normal or constitutive aspects of metallothionein synthesis and turnover in mammals, and correlations also were made with non-mammalian organisms. There is a growing body of evidence, therefore, that metallothioneins are indeed involved in the regulation of metal metabolism.

Recently it was demonstrated that blue crabs collected from unpolluted environments significantly alter tissue metal concentrations and the metal composition of metallothionein during the molt cycle (Engel, 1987). These studies clearly demonstrated that metallothionein in a marine crustacean is actively involved in normal physiological and biochemical processes of metal regulation at the cellular level that control growth and reproduction. Additionally, blue crab metallothionein also is associated with cellular metal detoxification and sequestration (Brouwer *et al.*, 1984; Engel and Brouwer, 1984).

Two series of experiments were performed to explore further the role of metallothionein in metal metabolism. The first series of experiments examined the effect of overwintering on the metal metabolism of the blue crab at both the tissue and cytosolic level. The second series of experiments measured the ability of metallothionein to donate copper for activation of apohemocyanin *in vitro*. In addition, we discuss how metallothionein and the metals bound to it relate to the physiological and biochemical changes that occur during molting. We also propose a model for the direct involvement of metallothionein as a metal donor in the synthesis of hemocyanin and zinc enzymes.

MATERIALS AND METHODS

All crabs used in these experiments were captured in the vicinity of Beaufort, North Carolina, by commercial fishermen. A group of ten intermolt (C₄) male blue crabs (*Callinectes sapidus*) were obtained in February 1986, and were maintained in the laboratory at ambient temperature and salinity (6°C, 30‰). After a week of acclimation, five crabs were taken for hemolymph and tissue samples. The remaining five crabs were held for an additional three weeks, and water temperature was allowed to increase to about 20°C in 10 days. During the three week period the crabs were fed chopped fish every other day. At the end of three weeks the remaining five crabs were killed and hemolymph and digestive gland samples were taken.

Tissue metal measurements

The concentrations of copper and zinc were determined in samples of digestive gland and hemolymph from individual blue crabs. The hemolymph samples were collected by severing the fifth pereopod at the meropodite and collecting the fluid in a polyethylene vial. A portion of the hemolymph was taken for metal analysis and the remainder was used for determination of hemocyanin concentration. The crabs were killed by removing the carapace, and the digestive gland was dissected out and used for total metal measurements and cytosolic metal determinations. The tissue

TABLE I

Amino acid composition of blue crab and lobster metallothionein (Residues/6500 Daltons)

	Blue crab			Lobster
	CdMT ^a gill	CdMT ^b digestive gland	ZnMT ^b digestive gland	CuMT ^c digestive gland
Cysteine	18	17	18	18
Asp/Asn	4	4	4	3
Thr	3	5	5	4
Ser	7	5	6	5
Glu/Gln	8	7	6	4
Pro	5	6	5	6
Gly	5	7	6	5
Ala	2	3	3	3
Val	1	1	1	—
Met	—	—	—	—
Ile	—	—	—	—
Leu	—	1	1	—
Tyr	—	—	—	—
Phe	—	—	—	—
His	1	—	—	—
Lys	7	7	7	8
Arg	1	1	1	1
	62	64	63	57

^a Brouwer *et al.* 1984.^b Brouwer unpub. results.^c Brouwer *et al.* 1986.

that was used for determination of cytosolic distribution of metals was frozen rapidly and stored in a freezer at -70°C .

Tissue samples used for metal analysis were oven dried at 100°C for 48 h and wet ashed with concentrated HNO_3 at 90°C . Residue was dissolved in 0.25 *N* HCl and concentrations of copper and zinc were measured using flame atomic absorption spectrophotometry. Preparative and measurement techniques were calibrated against the National Bureau of Standards, Oyster Reference Material #1566.

Apothemocyanin reconstitution experiments

We have shown that the digestive gland of the American Lobster, *Homarus americanus*, contains an abundant supply of copper-metallothionein (Engel and Brouwer, 1986). The amino acid composition of the purified metallothionein from lobster is similar to that of the blue crab (Table I). In view of this similarity, and the relative ease with which it can be isolated from the lobster, we have used lobster digestive gland as the source of copper metallothionein in our apothemocyanin reconstitution experiments.

Hemocyanin and copper metallothionein, to be used in copper transfer experiments, were prepared as described previously (Brouwer *et al.*, 1986). Hemocyanin concentration was calculated from the optical density at 280 nm, using $E_{1\text{cm}}^{1\%} = 14.3$ and a value of 75,000 for the molecular weight of a single oxygen-binding site carrying subunit (Nickerson and Van Holde, 1971). Apothemocyanin was prepared by mixing

hemocyanin in 50 mM Tris pH 8, 10 mM CaCl_2 , with an equal volume of buffer containing 20 mM KCN. To prepare hemocyanin samples with different amounts of bound copper, the protein was either incubated with KCN for 10 minutes at room temperature, or dialyzed for 30 minutes against 20 mM KCN, followed by removal of the KCN on Sephadex G-25. Reconstitution of apohemocyanin was performed by mixing the apoprotein with purified copper metallothioneins in 50 mM Tris pH 8, 10 mM CaCl_2 , in the absence of oxygen.

Copper insertion into the active site of the apoprotein was measured by fluorescence spectroscopy. Apohemocyanin was excited at 280 nm and the quenching of the tryptophan fluorescence, which accompanies copper incorporation, was monitored at 340 nm with a SPEX Fluorolog fluorescence spectrophotometer in the ratio mode. The concentration of functionally active oxygen binding sites was determined from the intensity of the copper-oxygen charge transfer band at 340 nm after addition of O_2 to the degassed incubation mixture.

RESULTS

Effect of overwintering on metal partitioning

Differences were observed in the concentrations of copper in hemolymph and digestive gland samples among the three groups of intermolt C_4 crabs that were examined (summer, 1985; winter-cold, 1986; and winter-warmed, 1986). In the hemolymph there was a correlation between the physiological condition of crabs and the concentrations of hemocyanin and copper (Fig. 1). Both summer and warmed hard crabs had hemocyanin and copper concentrations that were higher than the cold crabs, but only the difference between the copper concentrations in summer and cold hard crabs was significant ($P < .05$). Zinc concentrations did not change significantly ($P > .05$) among the three groups of crabs (Fig. 1), and did not appear to be positively correlated with hemocyanin concentration. In the digestive glands there was no significant difference ($P > .05$) in concentrations of copper between the summer and cold water crabs, but there was a significant ($P < .05$) increase in the crabs that were warmed (Fig. 2). Once again zinc concentrations did not show significant changes ($P > .05$). The large increase in copper concentration in the warmed crabs is correlated with the observed increase in hemocyanin in the hemolymph.

The elution profiles obtained after gel-permeation chromatography of the cytosol from digestive glands of cold and warmed crabs showed differences in metals bound to metallothionein. Among the cold water crabs four of five had metallothionein peaks that contained primarily zinc, while three of four (*i.e.*, one chromatographic sample lost) of the warmed crabs had metallothioneins that contained primarily copper (Fig. 3-II and III). Thus, the majority of cold water crabs had Cu/Zn ratios associated with metallothionein that were reminiscent of premolt animals (*i.e.*, high zinc low copper) while the majority of warmed crabs had patterns similar to those of summer intermolt crabs (*i.e.*, high copper low zinc) (Fig. 3-I). These data show that environmental conditions, physiological state, and feeding can affect tissue metal concentrations and the cytosolic distributions of copper and zinc in blue crabs.

Apohemocyanin reconstitution experiments

Removal of copper from the active site of hemocyanin results in an increase of the intrinsic tryptophan fluorescence of the protein (Fig. 4). This observation allowed us to make a distinction between copper insertion and formation of native functional

HEMOLYMPH

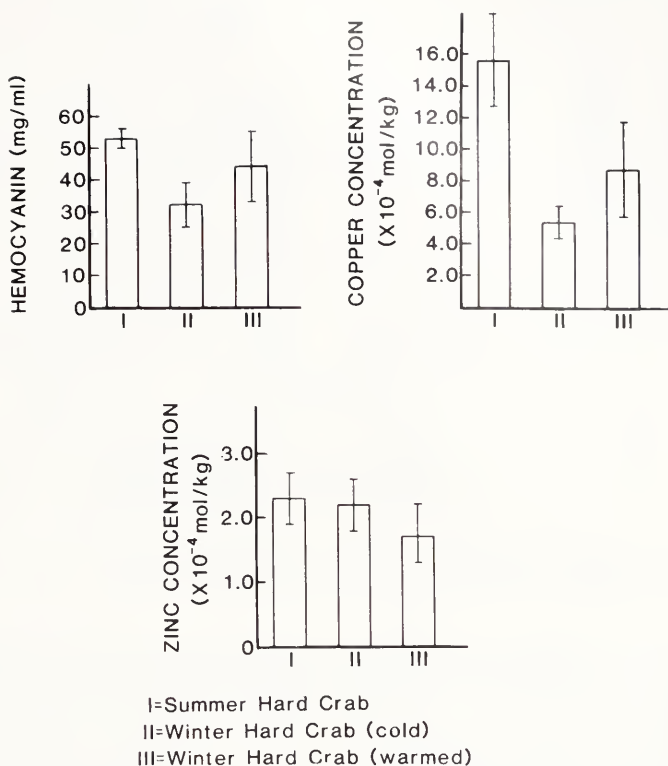


FIGURE 1. Concentrations of hemocyanin, copper, and zinc in the hemolymph of blue crabs collected in the summer (Engel, 1987) and winter. The winter crabs all were collected at the same time. Half (5) were sampled at ambient temperature (6°C) and the other half (5) were warmed to 20°C and fed fish every other day for three weeks. Each histogram represents a mean of five individual crabs plus or minus standard error of the mean.

oxygen binding sites. Both processes can be experimentally followed by fluorescence and absorbance spectroscopy as shown in Figure 5. The data demonstrated that incubation of apohemocyanin with copper metallothionein leads to fluorescence quenching before viable oxygen binding sites are formed, suggesting that copper insertion precedes the formation of biologically active oxygen binding sites (see Discussion).

DISCUSSION

As indicated earlier (Brouwer *et al.*, 1986), one of the proposed functions of copper metallothionein is as a Cu^{+1} donor for hemocyanin synthesis. The present experiment, with dormant and warmed crabs, shows that the predominance of copper on metallothionein among the warmed crabs is associated with the increased levels of hemocyanin in the hemolymph. Our earlier work with blue crabs also suggested a strong correlation between molting, copper metallothionein, and hemocyanin syn-

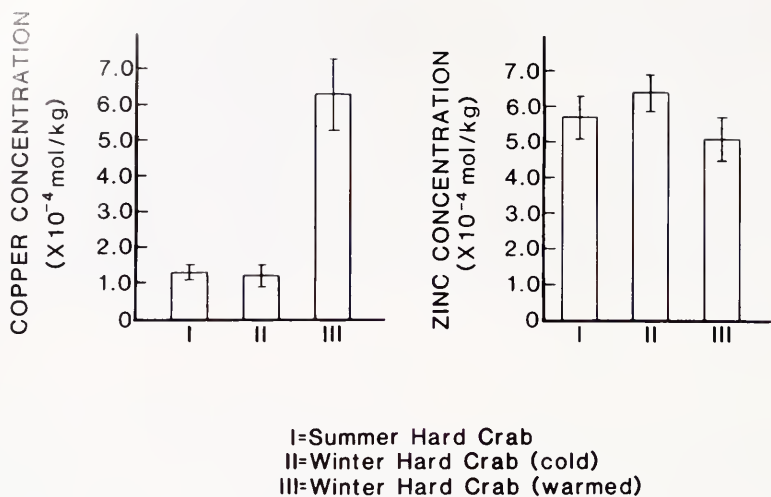
DIGESTIVE GLAND

FIGURE 2. Concentrations of copper and zinc in the digestive glands of blue crabs collected in the summer and winter. Further information on handling of the crabs is in Figure 1.

thesis (Engel, 1987). The studies reported in the present paper support that observation and demonstrate that environmentally induced changes and nutrition also can cause changes in the copper/zinc ratios associated with metallothionein. This observation is important because it further emphasizes the possible constitutive role of metallothioneins in normal metabolism.

In the following section we will develop a model of the regulation of copper and zinc partitioning in the blue crab, based on studies by us (Engel, 1987; also present paper), Soumoff and Skinner (1983), and Henry and Kormanik (1985).

The diagrams in Figure 6 display the physiological and biochemical processes involved in crustacean molting and the cyclic and chronological nature of these events. The first of these diagrams (Fig. 6A) depicts the relative duration of the different portions of the molt cycle. The actual timing of events is dependent upon both environmental temperature and the size of the crab (Johnson, 1980). This type of presentation emphasizes the fact that the most dramatic/traumatic changes in the crabs occur over a relatively short period of time. The changes in concentrations of copper and zinc associated with metallothionein are dramatic and provide further evidence as to the dynamic nature of the molting process (Fig. 6B). If it is assumed that metallothionein-bound copper and zinc are associated with metalloprotein and metalloenzyme synthesis, we can predict when hemocyanin and zinc enzyme synthesis occurs during the cycle (Fig. 6C, D). These predictions are tentative and will need to be confirmed in future studies, since there are no direct data available in the literature on these aspects of crustacean physiology.

Copper metallothionein levels during the molt cycle are related directly to hemocyanin concentrations in hemolymph, and inversely to ecdysteroid concentrations in the hemolymph (Fig. 7). The decrease in digestive gland copper metallothionein is

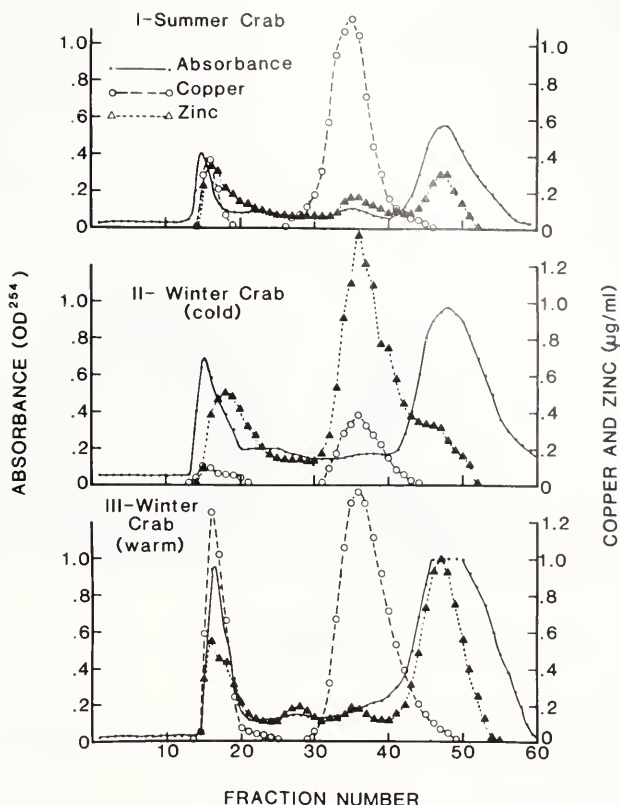


FIGURE 3. Sephadex G-75 elution profiles of digestive gland cytosol prepared from blue crabs collected during the summer and winter. For further information on the crabs see Figure 1. Protein separations were made using 60 mM Tris buffer, pH 7.9 with 2 mM β -mercaptoethanol with a 2.6×60 cm column at a flow rate of 30 ml/h in all three groups of crabs (I, II, III).

correlated with an increase in ecdysteroid titer in blue crab hemolymph (Soumoff and Skinner, 1983). The ecdysteroid concentrations followed the same general pattern for males and immature females throughout the molt cycle. After molt the ecdysteroid level decreases rapidly with concomitant decreases in hemocyanin concentrations. Coincident with these decreases is an increase in copper metallothionein during the A_2 and B_1 stages, followed by an increase in hemocyanin during B_1 . Such interrelationships suggest a possible association between the molting hormone ecdysteroid, and the regulation of hemocyanin synthesis and levels of cytosolic copper. It is relevant to emphasize that synthesis of constitutive metallothioneins in mammals is under the control of steroid hormones (Karin *et al.*, 1980 a, b; Karin *et al.*, 1981). No such information exists for the invertebrate metallothioneins. The effect of the molting hormone 20-hydroxyecdysone on metallothionein synthesis in the blue crab is presently under investigation.

Comparisons of zinc metallothionein (Engel, 1987) and ecdysteroid concentrations (Soumoff and Skinner, 1983) and carbonic anhydrase activity (Henry and Kormanik, 1985) during the molt cycle suggest an inter-relationship between these three

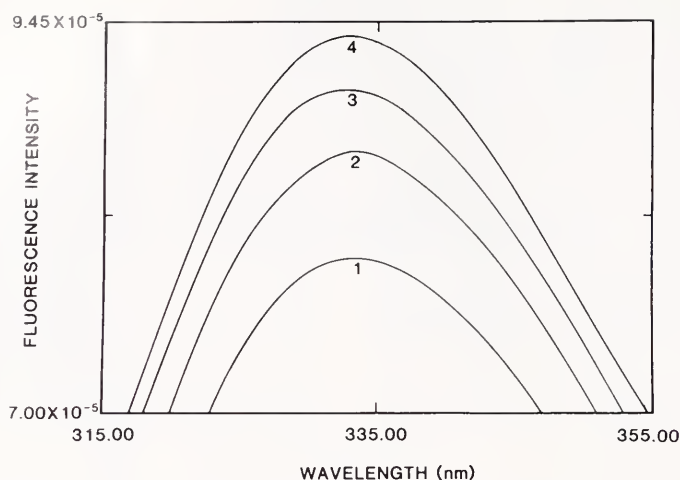


FIGURE 4. Fluorescence intensity of deoxygenated lobster hemocyanin in 50 mM Tris pH 8.0 + 10 mM CaCl_2 as a function of the percentage copper remaining in the active site after dialysis against 20 mM Cyanide for 0, 5, 10, and 30 min. (1) 100%, (2) 60%, (3) 30%, (4) 8%. Excitation is at 280 nm.

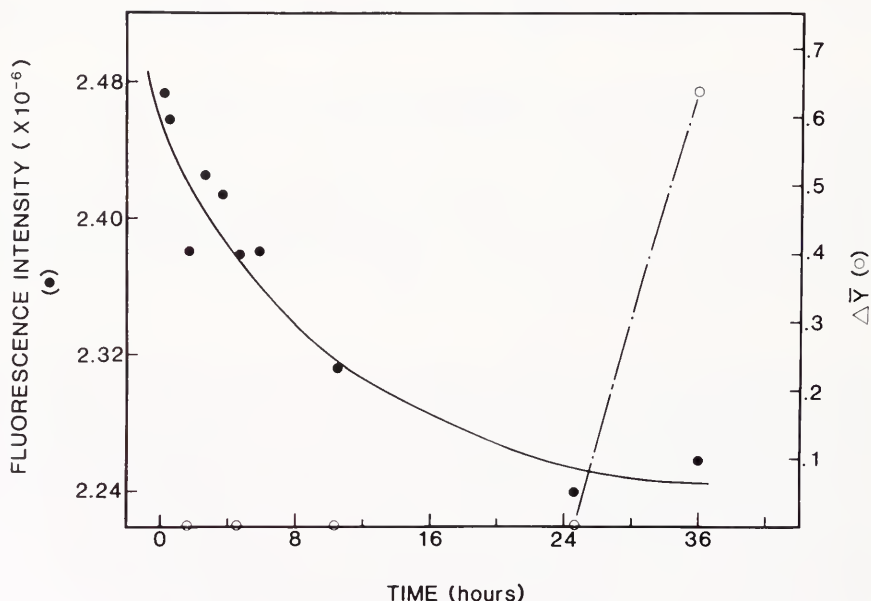


FIGURE 5. Change in fluorescence intensity and oxygen binding capacity (ΔY) of partial apohemocyanin ($5 \mu\text{M}$) in 50 mM Tris pH 8.0 + 10 mM CaCl_2 still containing 35% of its original copper, as a function of incubation time with copper-metallothionein ($10 \mu\text{M}$ Cu) in the absence of oxygen. The fluorescence change (copper insertion) precedes the formation of viable oxygen binding sites.

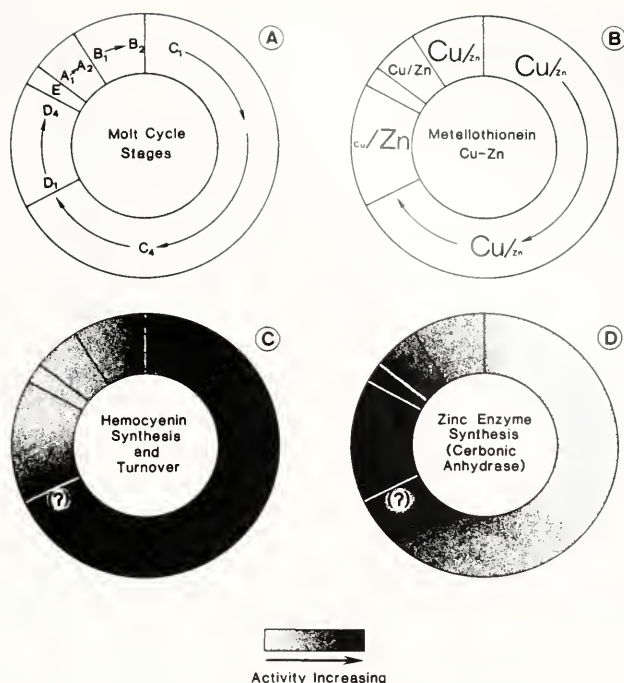


FIGURE 6. Diagrammatic representation of the physiological and biochemical events occurring during the molt cycle of the blue crab. (A) the molt cycle of the blue crab with the duration of each portion indicating time. The designations of the molt stages are: C₁ → C₄, hard crab; D₁ → D₄; premolt; E, ecdysis; A₁–A₂, soft crab; B₁–B₂, papershell crab (Mangum, 1985). (B) The relative concentrations of copper and zinc on metallothionein are represented by the size of the copper, Cu and zinc, Zn symbols. (C) and (D) These two figures represent predicted hemocyanin and zinc enzyme synthesis activities generated from previously collected data (Engel, 1987). The degrees of shading are indications of the proposed activities of the biochemical pathways involved in hemocyanin synthesis and turnover, and zinc enzyme synthesis (carbonic anhydrase).

components (Fig. 8). During the premolt period (D₁–D₃) when both zinc metallothionein and ecdysteroid are at their peaks, the new epidermis is being synthesized beneath the existing exoskeleton. At molt both zinc metallothionein and ecdysteroid decrease, and between stages A₁ and A₂ there is an abrupt increase in carbonic anhydrase activity in the newly formed exoskeleton epidermis (Henry and Kormanik, 1985). This rapid increase, which occurs over a period of hours, suggests that the enzyme may be synthesized and present in the new epidermis as an apo-protein, and is not activated by zinc until after molt. Even though the decrease in zinc-metallothionein occurs in the digestive gland and the increase of carbonic anhydrase in the epidermis, these two events may be linked. Possibly some of the zinc bound to metallothionein at the time of molt could be mobilized via the hemolymph to activate the apo-carbonic anhydrase. This hypothesis is attractive since preliminary results from our laboratory have shown the release of zinc from zinc metallothionein during stages A₁ and A₂ (D. W. Engel, unpub. data).

The proposed cycles for copper and zinc-metallothionein (Figs. 7, 8) are speculative, but they are based upon the best available information on the physiological and

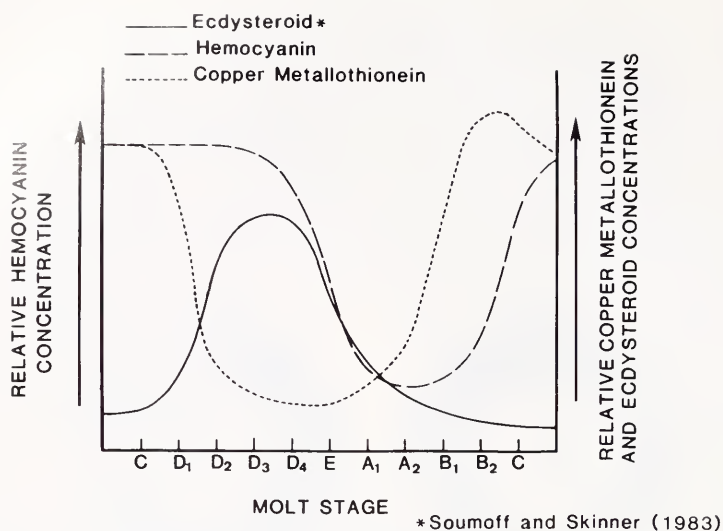


FIGURE 7. A diagrammatic representation of the processes involved in hemocyanin synthesis and turnover, and the interactions between ecdysteroids and copper metallothionein. The data on hemocyanin and copper metallothionein concentrations are from Engel (1987) and for ecdysteroid from Soumoff and Skinner (1983).

biochemical events controlling metal partitioning during molt. These changes are reproducible, and our experiments concerning the effects of thermal changes on metal distributions give further support to the hypothesis that metallothionein is a constitutive metal-binding protein in blue crabs.

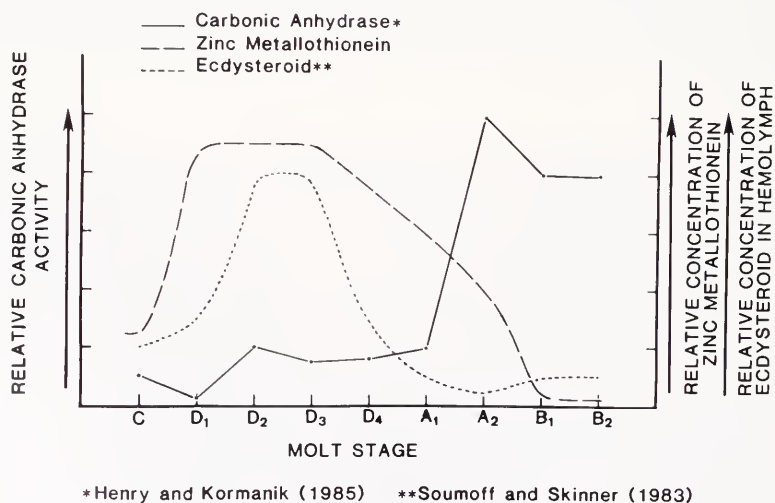


FIGURE 8. A diagrammatic representation of the processes involved in the synthesis of zinc-dependent enzymes and in particular carbonic anhydrase, and how zinc metallothionein and ecdysteroid interact to affect enzyme activity. The data on zinc metallothionein are from Engel (1987), on ecdysteroid from Soumoff and Skinner (1983), and carbonic anhydrase from Henry and Kormanik (1985).

The events and processes described here and in an earlier publication (Engel, 1987) do not address the question of control of the molt cycle and metal turnover. During the molt cycle there are pronounced changes in the cytosolic distribution and tissue concentrations of metals and accompanying changes in the hemolymph ecdysteroid concentrations. Studies by Singer and Lee (1977) suggest that the hemolymph hormonal levels are modulated by changing MFO (mixed function oxygenases) activity in the antennal gland. These authors demonstrated that MFO activity in the gland also varies with stages of the molt cycle. This activity is negatively correlated with the ecdysteroid levels (Soumoff and Skinner, 1983), suggesting that the MFO system controls steroid/hormonal concentrations in molting blue crabs, which in turn may affect metal partitioning as described in this paper.

Further evidence for metallothionein's metal regulatory function comes from the *in vitro* hemocyanin reconstitution experiments. Apohemocyanin can only be reconstituted with Cu^{+1} (Konings *et al.*, 1969; Lontie and Witters, 1973). Since copper binds to metallothionein as Cu^{+1} , and since copper metallothionein levels and hemocyanin biosynthesis seem to be linked *in vivo*, we initiated a study of hemocyanin-copper metallothionein interaction *in vitro*. The data presented in Figure 4 show that the intrinsic tryptophan fluorescence of lobster hemocyanin strongly depends on the amount of Cu^{+1} bound to the active site. This is in line with the observations that several crustacean hemocyanins contain tryptophan residues in close proximity to the binuclear copper site (Gaykema *et al.*, 1984; Linzen *et al.*, 1985). This property allowed us to make a distinction between Cu^{+1} incorporation into the active site of apohemocyanin and the formation of native functional oxygen binding sites. It is evident from Figure 5 that the quenching of tryptophan fluorescence, observed when apohemocyanin is incubated with copper metallothionein, precedes the formation of biologically active oxygen binding sites. This strongly suggests that the copper transfer process is followed by a slow reordering of the tertiary structure of the copper sites to the native configuration. Similar sequences have been demonstrated for the reconstitution process of many Cu^{+2} proteins where binding of copper to the active site is followed by a slow return of the protein to its biologically active state (Kertesz *et al.*, 1972; Morpurgo *et al.*, 1972; Rigo *et al.*, 1978; Marks *et al.*, 1979; Blaszak *et al.*, 1983). This observation may also explain why reconstitution of apohemocyanin with copper metallothionein can only be accomplished under anaerobic conditions (Brouwer *et al.*, 1986). The Cu^{+1} in the distorted sites is not capable of combining reversibly with oxygen. Interaction of oxygen with Cu^{+1} under these conditions results in oxidation of metal. These Cu^{+2} -sites will not bind oxygen and are lost for detection by absorbance spectroscopy. This hypothesis is presently under further investigation.

The studies described in this paper have demonstrated that marine crustacea are excellent model systems to study the role of metallothionein in copper/zinc metabolism on an organismal, cellular, and molecular level. Only when this function of metallothionein is fully understood will it be possible to assess its value as a metal-detoxifying protein.

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FREE D-AMINO ACIDS IN THE TISSUES OF MARINE BIVALVES

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ABSTRACT

Seventeen species of marine bivalves were surveyed for the presence of free D-alanine, D-aspartate, and D-valine in their tissues. D-aspartate was found in several species in concentrations approaching those of L-aspartate. D-alanine was detected—particularly in lucinid and vesicomyid clams—at levels exceeding manyfold those of L-alanine. D-valine was absent in all cases. A test of a hydrolysate of bulk soluble proteins of *Lucinoma aequizonata*, a species characterized by extremely high levels of D-alanine, showed no major incorporation of D-alanine into proteins. The implications of these results, for previously published analytical data and for human nutrition, are discussed.

INTRODUCTION

D-amino acids generally are viewed as natural oddities. They are usually not found in proteins, and they occur only occasionally in sizable quantities, either freely dissolved, or incorporated into peptides and metabolites in the tissues of animals and plants (Robinson, 1976; Bodanszky and Perlman, 1969; Corrigan, 1969). However, it has been suggested that the D-isomers of amino acids were as common as the L-forms in ancient prebiotic times on earth. Since the L-isomers were used exclusively by various life forms, they were removed from this natural equilibrium. The supply of L-amino acids was maintained by chemical racemization from the D-isomers left behind (Aono and Yuasa, 1977). Even today relatively large amounts of D-amino acids can be identified in oceanic waters, where apparently they have been formed by chemical racemization from the large pool of dissolved L-amino acids (Lee and Bada, 1977).

Widespread attention was first focused on D-amino acids when they were proposed to be causes and indicators of cancer. Proteins in tumor tissues were thought to contain high levels of D-glutamic acid, thus distinguishing them from normal tissue (Koegl and Erxleben, 1939). This theory was rejected, however, after years of controversy (Miller, 1950).

Recently, the occurrence of D-aspartate instead of the L-form in some proteins has been a focus of investigation for molecular repair mechanisms (McFadden and Clarke, 1982). It was reported that methylated aspartyl residues in erythrocyte membrane proteins had been converted to the D-form. According to the theory, the appearance of D-aspartate in proteins is a first sign of degradation. These proteins are either tagged for disposal, or the D-aspartyl residue can be reversed to the L-form after methylation.

A similar mechanism, time-dependent chemical racemization of aspartate within

proteins with a low turnover rate, like eye lens or dental proteins, has been used to date these proteins by measuring the ratio of the D- to the L-form of aspartate (Masters, 1983; Bada and Brown, 1980). Exactly the same principle of racemization has also been used extensively to determine, with great accuracy, the ages of fossils, since the normally present L-aspartate racemizes chemically at a constant rate after the death of an organism (Bada and Schroeder, 1975).

D-aspartate may also be a neurotransmitter (Wiklund *et al.*, 1982). Presently, it is being used experimentally as a non-metabolizable replacement for L-glutamate and L-aspartate in neurotransmitter research, since it can use the same uptake sites (Drejer *et al.*, 1983; Taxt and Storm-Mathisen, 1984).

Since finding that the artificial sweetener aspartame produces D-aspartate when heated (*e.g.*, during cooking) another line of research has been initiated (Boehm and Bada, 1984). Thus humans may be exposed nutritionally to considerable amounts of a D-amino acid due to increased consumption of aspartame worldwide.

Only a few publications have focused on the metabolic role of free D-amino acids in animal and plant tissue. D-alanine is present in some molluscs (Matsushima *et al.*, 1984) and crustaceans (D'Aniello and Giuditta, 1980), and it has been demonstrated to be synthesized during anaerobic metabolism in annelids (Felbeck, 1980; Schoettler *et al.*, 1983). Recently, a study of D-amino acids, as indicated by the reaction with D-amino acid oxidase, in a variety of marine invertebrates was published (Preston, 1987a). D-amino acids were found in 18 of the 43 species of the 8 phyla surveyed. The presence and metabolism of D-aspartate has been investigated in the tissues of the bivalve *Solemya reidi* (Felbeck, 1985) and of some cephalopods (D'Aniello and Giuditta, 1977, 1978). In all cases, the D- and the L-forms were present in about equal concentrations. The concentrations of D- and L-alanine in the polychaete *Arenicola marina* are approximately the same, and the concentrations of the two isomers increase similarly in response to metabolic stress (Felbeck, 1980; Schoettler *et al.*, 1983). The bivalve *Solemya reidi* takes up D-aspartate from environmental seawater and metabolizes it just as quickly as it does the L-form. Initially the D-form is converted into the L-isomer before further metabolism takes place (Felbeck, 1985). The uptake and metabolism of D-alanine from seawater has been described recently for coelomocytes of the annelid *Glycera dibranchiata* (Preston, 1987b).

Marine invertebrates commonly have extremely high concentrations of free amino acids which, in the event of osmotic stress, form the largest share of the pool of intracellular osmolytes (Bishop *et al.*, 1983). Therefore, D-amino acids in this pool might serve as important metabolic reserves, sinks, or regulatory factors.

Several investigators have described either isolated occurrences of individual D-amino acids in some invertebrates or have measured the unspecified presence of D-amino acids. No known publication has surveyed organisms for individual D-amino acids. The recent availability of chromatographic screening techniques for some amino acid isomers prompted our investigation of a number of marine bivalves for the presence of specific D-amino acids. We chose to study the Bivalvia because their physiology is well known, a variety of species is readily available, and their tissues contain high concentrations of free amino acids.

MATERIALS AND METHODS

Animals

Animals were purchased live at fish markets or collected from a variety of locations (Table I). The animals from the Santa Barbara channel were collected shipboard

TABLE I

Collection areas of animals

Animal	Collection area
<i>Bathymodiolus thermophilus</i>	Pacific, Galapagos hydrothermal vents
<i>Calyptogenia elongata</i>	Pacific, Santa Barbara channel
<i>Chione californiensis</i>	Pacific, Gulf of California
<i>Chione stutchburyi</i>	Fish market, San Diego, California
<i>Codakia obicularis</i>	Atlantic, Bahamas, intertidal
<i>Codakia tigrina</i>	Fish market, Philippines
<i>Corbicula fluminea</i>	Fish market, San Diego
<i>Crassostrea virginica</i>	Fish market, San Diego
<i>Hiatella pholadis</i>	Pacific, La Jolla, California
<i>Hinnites multirugosus</i>	Pacific, San Diego
<i>Lima hemphilli</i>	Pacific, San Diego
<i>Lucinoma acquionata</i>	Pacific, Santa Barbara channel
<i>Mercenaria mercenaria</i>	Fish market, San Diego
<i>Modiolus capax</i>	Pacific, San Diego
<i>Mytilus edulis</i>	Fish market, San Diego
<i>Solemya reidi</i>	Pacific, Santa Monica Bay
<i>Tapes japonica</i>	Fish market, San Diego

by otter-trawl. *Solemya reidi* was collected shipboard in Santa Monica Bay by Van Veen grab. Most other species were collected by divers and were maintained alive in flow-through seawater tanks, at approximate *in situ* temperatures, for a maximum of ten days before being sacrificed. *Bathymodiolus thermophilus* specimens were collected by the submarine DSRV "Alvin" during a cruise to the Galapagos hydrothermal vents. The animals were frozen upon retrieval. *Codakia tigrina* was purchased alive at a fish market in the Philippines and then shipped by air freight in 70% alcohol.

Sample preparation

To account for the presence of symbiotic bacteria in the gills of some of the bivalves used in this study (Felbeck *et al.*, 1981; Felbeck, 1983), all bivalves were opened, and the gills were removed and analyzed separately from the remaining soft parts.

The tissue, frozen with liquid nitrogen, was first pulverized in a mortar. The homogenization was then completed in 1 *N* HClO₄ with an Ultra-Turrax homogenizer. The homogenate was centrifuged at 12,000 × *g* for 15 min, and the supernatant was neutralized with 3 *M* KHCO₃. The resulting precipitate was removed by centrifugation. An aliquot of this extract was derivatized with o-phthaldialdehyde (OPA) and N-acetyl-L-cystein (NAC), according to the method described by Aswad (1984). The amino acid isomers were then separated on a C18 reverse phase column with a gradient of 50 mM sodium acetate, pH 5.8, containing 8% methanol (Sol. A) to methanol (Sol. B). The gradient was (in % of solution B): 0 min, 0%; 4 min, 0%; 10 min 25%; 20 min 27%; 34 min, 52%; and 50 min, 52%. Using this gradient—which was modified from the one described by Aswad (whose sole purpose was to separate D- and L-aspartate)—the two alanine and valine isomers could also be separated completely.

Using a Gilson Datamaster integrator, standards for the D- and L-isomers of aspartate, valine, and alanine were used to determine standard response curves. When samples were analyzed, the area under each individual peak was used to determine concentration and, subsequently, the ratio of the individual stereoisomers.

To determine whether D-alanine was present in the proteins of *L. aequizonata*, tissue of a whole animal was homogenized in distilled water with an Ultra Turrax. After centrifugation, the pellet was twice resuspended and rehomogenized in water. The combined supernatants were dialyzed against multiple changes of distilled water for five days to remove all free amino acids. The resulting solution of mixed soluble proteins of *L. aequizonata* was then precipitated with perchloric acid, centrifuged, and the pellet hydrolyzed overnight with HCl. The hydrolyzate was then analyzed for D-amino acids as described above.

RESULTS

Significant concentrations of D-aspartate and D-alanine and their L-isomers were detected (Table II); no D-valine was found. All Lucinidae showed high concentrations of D-alanine—concentrations much higher than those of L-alanine. D-alanine also was detected in *Mercenaria mercenaria*, both species of *Chione*, *Hinnites giganteus*, *Lima hemphilli* (gills), *Bathymodiolus thermophilus*, *Crassostrea virginica* (gills), *Tapes japonica*, *Hiatella pholadis*, and *Corbicula fluminea*. In all of these species, the concentration ratios of D- to L-isomer was below one. In the Mytilidae *Mytilus edulis* and *Modiolus capax*, no D-alanine was detected, but D-aspartate was found in concentrations approaching those of the L-isomer. D-aspartate was also detected in the gills of *Bathymodiolus thermophilus*.

No D-alanine was detected in the hydrolyzed soluble protein fraction of *Lucinoma aequizonata*.

Because amino acid levels among individual animals of the same species are typically highly variable, we did not attempt to establish average concentrations for a large number of bivalves but instead focused on the presence of D-amino acids. We postulate that the detection of D-amino acids in any individual organism is significant for the species in general.

DISCUSSION

The lucinids contained the highest D- to L-ratio of alanine. The extremely high level of free alanine in *Codakia obicularis* tissues has been measured only by ion-exchange amino acid analysis and, therefore, has been attributed entirely to "generic" alanine acting as an osmoregulatory agent or an end-product of anaerobic metabolism (Berg and Alatalo, 1984). In fact, most of this alanine is in the D-form, prompting us to question the function of the D-alanine in this bivalve as well as in all lucinid clams. One possibility is that the D-alanine is entirely made by the symbiotic bacteria inside the cells of the gill. Gram negative bacteria, like the symbiotic species found in the gill (see Schweimanns and Felbeck, 1985, for review), often contain D-alanine in their cell walls (Katz and Derrain, 1977). Therefore, extraction of the cell wall could yield significant amounts of the D-isomer of alanine. In addition, these bacteria are thought to provide a major share of the bivalves' nutritional needs by fixing CO₂ from the seawater and transferring reduced organic compounds, possibly including D-alanine, to the host. It is unlikely that the D-amino acids originate in bacteria, however, since tissues lacking bacteria have a D- to L-isomer ratio similar to that of gills densely populated with bacteria. If the bacteria produce and export D-alanine, then the gill preparations should show a larger share of the D-isomer. In addition, some bivalve species (Table II) without symbiotic bacteria also have high concentrations of D-alanine.

Another peculiar aspect of the large proportion of D-alanine in the free amino

TABLE II

Concentrations of D- and L-amino acids in the tissues of marine bivalves

Animal	Tissue	n	L-alanine ($\mu\text{mol/g}$ fw) ($\bar{x} \pm \text{SD}$)	D-alanine ($\mu\text{mol/g}$ fw) ($\bar{x} \pm \text{SD}$)	Ratio D/L	L-aspartate ($\mu\text{mol/g}$ fw) ($\bar{x} \pm \text{SD}$)	D-aspartate ($\mu\text{mol/g}$ fw) ($\bar{x} \pm \text{SD}$)	Ratio D/L
SOLEMYIDAE								
<i>Solemya reidi</i>	foot	2	14.2 $\pm$ 6.9	0.1 $\pm$ 0.1	0.01	13.3 $\pm$ 2.4	12.4 $\pm$ 1.6	0.93
	gill	2	3.9 $\pm$ 0.7	1.4 $\pm$ 0.6	0.36	7.6 $\pm$ 2.0	5.4 $\pm$ 2.2	0.71
MYTILIDAE								
<i>Mytilus edulis</i>	foot	2	4.4 $\pm$ 0.2	—	—	4.6 $\pm$ 1.8	1.4 $\pm$ 0.5	0.3
	gill	2	2.8 $\pm$ 0.9	—	—	3.9 $\pm$ 0.8	3.5 $\pm$ 0.7	0.9
<i>Modiolus capax</i>	foot	2	2.7 $\pm$ 1.4	4 $\pm$ 5.7	1.5	6.9 $\pm$ 2.7	2.6 $\pm$ 0.01	0.38
	gill	1	0.5	0.7	1.4	2.9	2.3	0.79
<i>Bathymodiolus thermophilus</i>	foot	1	12.3	0.9	0.08	2.4	—	—
	gill	1	11.4	3.2	0.28	2.2	0.7	0.31
OSTREIDAE								
<i>Crassostrea virginica</i>	mantle	1	19.4	—	—	3.5	—	—
	gill	2	9.9 $\pm$ 2.6	1.1 $\pm$ 0.1	0.12	5.3 $\pm$ 0.5	—	—
PECTINIDAE								
<i>Hinnites multirugosus</i>	foot	2	1.4 $\pm$ 1.2	1.1 $\pm$ 0.9	0.79	1.0 $\pm$ 1.3	0.4 $\pm$ 0.6	0.4
	gill	2	0.5 $\pm$ 0.2	0.7 $\pm$ 0.9	1.4	0.5 $\pm$ 0.2	0.2 $\pm$ 0.2	0.4
LIMIDAE								
<i>Lima hemphilli</i>	gill	2	1.0 $\pm$ 0.5	0.5 $\pm$ 0.3	0.5	0.8 $\pm$ 0.1	—	—
VENERIDAE								
<i>Tapes japonica</i>	foot	1	13.4	11.2	0.84	15.5	—	—
	gill	1	4.7	3.5	0.74	1.7	0.2	0.12
<i>Chione californiensis</i>	foot	1	3.0	2.1	0.7	14.6	—	—
	gill	3	3.8 $\pm$ 2.6	1.8 $\pm$ 1.5	0.47	4.5 $\pm$ 0.9	—	—
<i>Chione stutchburyi</i>	foot	1	4.6	2.0	0.44	6.6	—	—
	gill	2	3.3 $\pm$ 1.5	1.3 $\pm$ 0.7	0.39	3.3 $\pm$ 2.8	—	—
<i>Mercenaria mercenaria</i>	foot	2	17.7 $\pm$ 3.1	16.2 $\pm$ 1.9	0.92	7.8 $\pm$ 3.5	—	—
	gill	3	4.2 $\pm$ 2.2	2.8 $\pm$ 1.4	0.67	4.1 $\pm$ 1.4	—	—
CORBICULIDAE								
<i>Corbicula fluminea</i>	foot	2	1.9 $\pm$ 0.4	0.9 $\pm$ 1.2	0.47	0.7 $\pm$ 0.2	—	—
	gill	3	3.8 $\pm$ 0.8	0.6 $\pm$ 0.1	0.16	0.4 $\pm$ 0.3	—	—
HIATELLIDAE								
<i>Hiatella pholadis</i>	gill	2	4.4 $\pm$ 1.0	1.4 $\pm$ 0.2	0.32	2.3 $\pm$.01	—	—
VESICOMYIDAE								
<i>Calyptogena elongata</i>	foot	1	1.1	11.3	10.45	11.5	—	—
	gill	1	0.3	2.7	9.0	1.4	—	—
LUCINIDAE								
<i>Codakia obicularis</i>	foot	1	13.5	187.2	13.9	0.3	0.2	0.67
	gill	1	2.4	26.6	11.1	0.3	0.1	0.33
<i>Codakia tigerina</i>	foot	1	0.4	22.4	56.0	0.6	0.1	0.17
	gill	1	0.4	21.5	53.7	0.4	—	—
<i>Lucinoma acquizonata</i>	foot	1	3.8	84.2	22.2	0.4	0.3	0.8
	gill	3	3.1 $\pm$ 0.5	26.1 $\pm$ 4.1	8.4	1.0 $\pm$ 0.9	0.1	0.1

The levels are given in $\mu\text{mol}/\text{gram}$ fresh weight with the standard deviation. When only gills were tested, the foot was too small to be easily dissected and analysed. ("—" below 0.1 $\mu\text{mol}/\text{g}$ fw)

acids of Lucinidae is that the enzyme most commonly responsible for the formation of D-amino acids—amino-acid racemase—would cause an equal distribution between the two isomers (Barman, 1969). The fact that up to 98% of the free alanine pool is in the D-form suggests that: (a) another specialized enzyme is responsible for the metabolism of the D-isomer; and (b) the D-isomer is not metabolized after conversion to the L-form by a racemase, but used separately. Aside from the lucinid clams, however, other examples were found where the ratio of D- to L-alanine was lower: below one. Here we assume that a racemase interconverts the two isomers.

The occurrence of D-aspartate can be explained more easily by the presence of a

racemase, for the maximal ratio of the D- to the L-isomer was around one. Indeed, this specific racemase has already been demonstrated in *Solemya reidi* (Felbeck, 1985). The aspartate-racemase does not catalyze the conversion of alanine.

In spite of the high D-alanine concentration in *Lucinoma aequizonata*, no detectable quantities of D-alanine were found in the proteins of this animal. Therefore, the selection for the L-isomer of alanine in protein synthesis is significant. Since the method used only provides a crude overview of a selected group of proteins—those soluble in distilled water—we cannot exclude the possibility that some minor fraction of the soluble or insoluble proteins would include D-alanine; neither of these would have been detected by the method used.

Wide ranging surveys for the presence of D-amino acids are rarely in the literature. The review article by Corrigan (1969) and Preston's (1987a) recent results are the only known examples. Certainly, one reason is that simple, quick methods to determine the concentrations of D- and L-isomers of individual amino acids have been published only recently. Before this, either both isomers were detected as a sum (e.g., in HPLC with OPA/mercaptoethanol derivatization or with the classical ion exchange amino acid analyzer) or just the L-isomer was detected in typically stereospecific enzymatic determinations. Since it was always assumed that no D-amino acids were present, the results obtained by these methods were taken as representative for "all" amino acids. The D-amino acids concentrations found by Preston (1987a) were obtained unspecifically with a test using D-amino acid oxidase and, therefore, were only applicable as indicator of the general presence of most D-amino acids (D-aspartate and D-glutamate do not react with the D-amino acid oxidase).

Our survey includes the amino acids alanine and aspartate, both of which are commonly found in high concentrations in marine invertebrates, and shows the frequent occurrence of both stereoisomers.

This result is significant for "standard" experimental research organisms like *Mytilus edulis* (Bishop *et al.*, 1983). In this species, the pool of free aspartate is used as an initial substrate for anaerobic metabolism (see de Zwaan and Putzer, 1985, for review). Whenever the concentration of this amino acid was tested using enzymatic methods, only about half of the available amino acid was detected; *i.e.*, the pool of aspartate was actually higher than measured. This may explain the apparent lack of enough initial substrate for anaerobic energy metabolism, as recently reviewed by de Zwaan and Putzer (1985). Similarly, in other organisms such as the polychaete *Arenicola marina*, the initial depletion of the (enzymatically measured) L-aspartate is not large enough (Felbeck, 1980; Schoettler *et al.*, 1983). We think it is possible that D-aspartate, as well as the L-isomer, occurs in *Arenicola*, and that it serves there as additional substrate not detected by enzymatic analysis which after rapid racemization also can be used as metabolic substrate.

We conclude that many published results where amino acid levels in invertebrates have been used as indicators for metabolic pathways, or to calculate metabolic rates, will have to be reassessed because D-amino acids may be present in the tissues used.

Currently, we can only speculate what the metabolic role of D-amino acids is in marine invertebrates. Amino acids are usually used as osmolytes in the tissues of marine invertebrates and, therefore, are often present in very high concentrations (Bishop, 1983; Yancey *et al.*, 1983). The exchange of part or most of the L-isomer for the D-isomer may influence regulatory mechanisms involving these amino acids. Glutamate-pyruvate-transaminase is inhibited by high levels of L-alanine (Barman, 1969); the D-isomer may not have this effect on this enzyme.

Finally, large quantities of free D-amino acids in tissues of common marine bi-

valves may affect human health. Some of our test species were obtained from commercial fish markets. For example, large quantities of D-alanine were found in *Codakia tigerina* specimens bought in a fish market in the Philippines. These fish are routinely consumed by humans. Even *Mytilus edulis*, one of the most common bivalves cultured and consumed in large quantities, contains a concentration of D-aspartate, equal to that of L-aspartate, which is usually between 3 and 14 $\mu\text{mol/g}$ fresh weight (de Zwaan and Putzer, 1985). Little research has been done on the metabolism of D-amino acids in humans or on the effect of long-term exposure to D-amino acids. D-amino acids can cause analgesia in humans, and some D-amino acids are powerful inhibitors of some enzymes involved in regular metabolic pathways (Koyuncuoglu and Berkman, 1982). The result presented in this paper—that some D-amino acids exist sometimes in extremely high concentrations in commonly consumed shellfish—should prompt a closer examination of the effects of D-amino acids on humans.

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TROPHOSOME ULTRASTRUCTURE AND THE CHARACTERIZATION OF ISOLATED BACTERIOCYTES FROM INVERTEBRATE-SULFUR BACTERIA SYMBIOSES

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ABSTRACT

Electron microscopy of trophosome tissue from the vestimentiferan tubeworm *Riftia pachyptila* clearly indicates that the bacterial symbionts are enclosed within animal cells (bacteriocytes). The structure of this lobular tissue is complex. Each lobule consists of an outer layer of trophochrome cells (devoid of symbionts, but with numerous pigmented granules), an inner region of bacteriocytes, and a central hemolymph space. Sulfur deposits within bacteria decrease in size and number with increasing distance of the bacteria from the hemolymph space. Bacteria located toward the center of the lobule appear smaller than those nearer the periphery, suggesting that metabolic and developmental gradients exist. Trophochrome cells and free bacteria were enriched from the trophosome of *R. pachyptila*.

A procedure is described for the isolation of bacteriocytes from gill tissue of the bivalves *Calyptogena magnifica* and *Lucina floridana*. Numerous bacteria reside in vacuoles within the bacteriocyte cytoplasm, as do large (5–10 micron), heterogeneous granules. Maximum CO₂ fixation rate at 20°C for bacteriocytes from *C. magnifica* is 13.2 nmoles CO₂/mg protein/h, compared to 21.6 nmoles CO₂/mg protein/h for *L. floridana* bacteriocytes. Fixation by bacteriocytes from *C. magnifica* is inhibited by sulfide, and to a lesser extent thiosulfate, at 0.1–1.0 mM. Thiosulfate increases CO₂ fixation two-fold in *L. floridana* bacteriocytes.

C. magnifica bacteriocytes incubated for 1 h in 0.5 mM sulfide maintain higher intracellular ATP concentrations (3.3 nmoles/million cells; 1.01 mM) than do control cells without sulfide (1.02 nmoles/million cells; 0.31 mM). These results and comparable observations suggest that the identities of exogenous sulfur compounds exploited for chemical energy by the symbiosis may depend on the structural integrity and organization of the experimental preparation.

INTRODUCTION

In symbiosis between sulfur bacteria and marine invertebrates, various metabolic features are critically dependent on the cellular integrity of each participant. To study these characteristics without disrupting cellular structure, we developed a procedure for isolating intact bacteriocytes (eucaryotic cells that contain large numbers of bacterial endosymbionts) from gill tissues of the hydrothermal vent clam, *Calyptogena magnifica*, and the shallow-water bivalve *Lucina floridana*, an inhabitant of seagrass beds. An ultrastructural description of the intact trophosome of the hydrothermal

vent tubeworm *Riftia pachyptila* (Pogonophora) provides new information about cellular arrangements and metabolic potentials in this symbiont-containing tissue. Finally, using bacteriocyte suspensions prepared from the bivalves, we measured intracellular ATP levels and the capacity for carbon fixation in the presence of various sulfur compounds.

The primary advantage of using bacteriocytes for metabolic studies is that the symbiotic bacteria are retained in their natural microenvironment. As a consequence, the bacteria receive chemical signals (sulfur compounds, dissolved gases, etc.) via the cytoplasm of the host cell. Furthermore, all bacteriocyte surfaces are in direct contact with medium constituents, so that the effects of slowly exchanging compartments such as connective tissue spaces are minimized. Thus individual bacteriocytes are considered functional symbiotic units, and their response to various stimuli quantified on a cellular basis.

Until now, isolated invertebrate cells have not been used to study physiological and biochemical relationships between sulfur oxidizing bacteria and the host. Rather, previous studies have focused on other levels of biological organization and complexity. Data have been obtained using (1) the intact symbiosis at the whole-organism level (e.g., Anderson, 1986; Arp *et al.*, 1984; Childress *et al.*, 1984; Felbeck, 1983, 1985; Fiala-Medioni *et al.*, 1986), (2) excised tissues (e.g., Cavanaugh, 1983; Dando *et al.*, 1985; Felbeck, 1983; Powell and Somero, 1983), (3) variously prepared homogenates of tissues (e.g., Felbeck, 1981; Felbeck *et al.*, 1981; Fisher and Childress, 1984; Fisher and Hand, 1984; Hand and Somero, 1983; Powell and Somero, 1985, 1986a), and (4) isolated bacteria and cellular organelles (e.g., Belkin *et al.*, 1986; Powell and Somero, 1986b). Depending on the degree of tissue disruption, significant variation was observed in the metabolism of sulfur compounds and the rates and characteristics of carbon fixation. For example, using homogenates of gill tissue from *C. magnifica*, Powell and Somero (1986b) reported that stimulation of ATP synthesis by sulfur compounds occurred only when bacteria contained therein were lysed.

Experimental preparations that maintain the bacteria in more biologically realistic surroundings offer new opportunities for assessing their metabolic potential. This possibility was the impetus for the present study. In addition to isolating bacteriocytes from gill tissue of *C. magnifica* and *L. floridana*, we isolated trophochrome cells (green pigmented cells) and free bacteria from trophosome tissue of *R. pachyptila*. Although electron micrographs presented herein indicate bacteriocytes within the trophosome, we were unsuccessful in isolating them intact from this source.

MATERIALS AND METHODS

Experimental animals and reagents

Specimens of *Riftia pachyptila* and *Calymene magnifica* were collected in March 1985 during the hydrothermal vent expedition to the Galapagos Rift with the submersible DSRV Alvin at the "Rose Garden" site (Hessler and Smithey, 1983). The live animals on board the RV Melville were handled as described by Powell and Somero (1986a). Tissue samples were dissected from specimens of *R. pachyptila* and *C. magnifica* typically within 3 h of their arrival on board ship. Tissue weights were determined with the motion compensated shipboard balance developed by Childress and Mickel (1980).

Specimens of the eulamellibranch bivalve *Lucina floridana* were collected from the sulfide-rich sediments of *Thalassia* and *Ruppia* seagrass beds in St. Joseph's Bay, Florida. Animals were maintained in the laboratory as described by Fisher and Hand (1984) for no more than three weeks prior to tissue dissection.

Hyaluronidase (Type I-S), collagenase (Type IV), DNAase I (Type IV), chymotrypsin (Type II), soybean trypsin inhibitor, and Percoll were purchased from Sigma Chemical Co. $\text{NaH}^{14}\text{CO}_3$ was obtained from New England Nuclear. All other chemicals were reagent grade. Solutions of sodium sulfide were prepared fresh before each experiment (Powell and Somero, 1986a) and maintained under nitrogen until use (1–3 h). To reduce mechanical damage to isolated cells, siliconized glassware was used in all steps described below, and all pipets were firepolished.

Tissue dissociation

C. magnifica gill tissue was placed on a chilled glass plate and minced into cubes varying in size from 0.5 mm to 2 mm. The tissue was rinsed briefly in $\text{Ca}^{++}\text{-Mg}^{++}$ free salt solution (CMF solution) (508 mM NaCl, 10 mM KCl, 8.7 mM NaHCO_3 , 28.6 mM Na_2SO_4 , 0.1 mM EGTA, 4 mM glucose, pH 7.2) to remove mucus. The tissue was then transferred to 50 ml flasks and incubated for 15 min at 20°C in 20 ml of CMF solution on a rotary shaker (110 cycles/min). This initial medium was replaced with 10 ml of artificial seawater (411 mM NaCl, 9.6 mM KCl, 54 mM MgCl_2 , 10.5 mM CaCl_2 , 8.8 mM NaHCO_3 , 23.6 mM Na_2SO_4 , glucose 4 mM, pH 7.2) containing hyaluronidase (400 U/ml), collagenase (500 U/ml), and chymotrypsin (70 U/ml), and the tissue was incubated for 1.5 h at 20°C. At the end of this period, the tissue was rinsed with CMF solution and incubated 15 min in 10 ml of CMF solution containing bovine serum albumin (1 mg/ml), trypsin inhibitor (0.4 mg/ml), and DNAase I (15 U/ml). The tissue in this solution was flushed 20–30 times through a siliconized Pasteur pipet, a procedure that released large numbers of cells. The cellular suspension was filtered sequentially through 250 micron and 100 micron nylon mesh (Tetko, Inc.; Elmsford, New York) to remove undissociated tissue.

Isolated cells from *Riftia* trophosome were prepared similarly, except the tissue incubation with enzymes was shortened to one hour at 20°C. The concentrations of enzymes were all reduced 50%, compared to the levels used for *C. magnifica*.

The procedure for dissociation of gill tissue from *L. floridana* differed from the above protocol for *C. magnifica* in several respects. The concentration of the artificial seawater was 40 ppt (470 mM NaCl, 11 mM KCl, 62 mM MgCl_2 , 12 mM CaCl_2 , 10 mM NaHCO_3 , 27 mM Na_2SO_4 , 0.1 mM EGTA, 5 mM glucose, pH 7.2), and the CMF solution consisted of 581 mM NaCl, 11.4 mM KCl, 10 mM NaHCO_3 , 32.7 mM Na_2SO_4 , 0.1 mM EGTA, 5 mM glucose, pH 7.2. The minced gill tissue was incubated for 15 min at 37°C in CMF solution, and the concentrations of enzymes used in the subsequent incubation (1 h at 37°C) were 50% of those used for *C. magnifica* tissue.

Cell isolation

The cellular suspension from *C. magnifica* gill was divided into two 5-ml portions, each of which was layered onto a Percoll gradient at 4°C. This gradient separated bacteriocytes from other cell types and from acellular and subcellular debris. Cellular suspensions of *R. pachyptila* trophosome were treated similarly. The 40-ml discontinuous gradient consisted of four steps of 10% (density, 1.042), 30% (1.065), 50% (1.089), and 70% (1.111) Percoll. Each step was prepared by adding appropriate amounts of Percoll and deionized water to 2 ml of a concentrated CMF stock (5X). The cells settled without centrifugation for 3 h at 4°C, and cells that had accumulated at each interface were collected and rinsed twice with artificial seawater to remove Percoll (which interferes with the assay for CO_2 incorporation).

The Percoll gradient was changed to 30%, 50%, 70%, and 90% (density, 1.120) for separation of *L. floridana* bacteriocytes. Each step was prepared in the 40 ppt CMF solution (final concentration).

Cell concentrations were determined with a hemocytometer. The distinguishing features used to identify bacteriocytes under light microscopy were their granular appearance, lack of cilia, and relatively large diameter (20 microns, *C. magnifica*; 40 microns, *L. floridana*).

Transmission electron microscopy

Isolated cells to be fixed for electron microscopy were transferred to Beem capsules and centrifuged at low speed ($500 \times g$) to concentrate the cells. The supernatant was removed, and glutaraldehyde (4% in 0.3 M PIPES buffer, pH 7.2 at room temperature) was layered over the cells. Intact tissue for fixation was dissected into small blocks (1 mm diameter) and placed in plastic specimen trays containing glutaraldehyde solution. After 30–60 min, the glutaraldehyde was removed, and the Beem capsule (or specimen tray) was filled with warmed agar (1.5% in 0.3 M PIPES, pH 7.2). After the agar solidified, the capsules were given three 15-min washes in buffer and then placed in 1% osmium tetroxide (prepared in 0.2 M potassium phosphate buffer, pH 7.4) for 2–3 h. The preparations were washed thoroughly with deionized water, dehydrated in a graded acetone series, and embedded in Spurr's low-viscosity media. All the steps above were completed on board ship. Sections were cut with a Sorvall MT 5000 Ultramicrotome and stained with 4% uranyl acetate followed by lead citrate. Cells were viewed with a Hitachi H-600 electron microscope.

CO₂ fixation studies

Isolated cells (100,000–500,000 for each assay) were incubated for up to 30 min in 0.5 ml of artificial seawater (pH 8.2) containing 1 microcurie of $\text{NaH}^{14}\text{CO}_3$, with and without various concentrations of sulfide and thiosulfate. All incubations were performed at 20°C. Incorporation of CO_2 was stopped by vigorously mixing 0.1 ml of 12 N HCl with each sample. Samples were transferred to plastic counting vials and heated for 2 h at 90°C. Radioactivity remaining in the acid soluble fraction was quantified with liquid scintillation counting. Values for duplicate samples stopped at time 0 were subtracted from all treatments.

ATP measurements

Experiments to determine the influence of sulfur compounds on cellular ATP levels were performed similarly to those above, but the radioactive bicarbonate was omitted from the incubation medium. At the end of the incubation, cells were sedimented with low speed centrifugation ($500 \times g$, 5 min) at 4°C, and the incubation medium was decanted. Cells were then resuspended in 0.5 ml of ice-cold 0.6 M perchloric acid and homogenized. The homogenate was neutralized (and perchlorate salts precipitated) with 0.15 ml of a solution containing 0.2 N KOH, 0.4 M imidazole, and 0.4 M KCl. The supernatant was initially stored in liquid nitrogen on board ship and later transferred to a -80°C freezer until ATP analyses could be performed.

ATP was measured with an enzyme-coupled fluorometric assay (modified from Lowry and Passonneau, 1972). All solutions were filtered through Gelman TCM-450 (0.45 micron) filters before use. The 1.2 ml assay mixture contained 50 mM Tris-HCl buffer pH 8.1, 1.0 mM MgCl_2 , 0.2 mM dithiothreitol, 0.05 mM NADP, 0.1 mM glucose, and 50 microliters of sample. First, 0.07 units of glucose-6-phosphate dehy-

drogenase were added to eliminate endogenous G-6-P, and then 0.34 units of hexokinase were added for the quantification of ATP. The excitation wavelength was 365 nm, and the emission monochromator was set at 460 nm. The increase in fluorescence was measured with a Turner Model 430 spectrofluorometer, and the fluorescence signal was adjusted so that 0.1 nmole of ATP gave a 25% full scale deflection.

Protein measurements

Total protein was analyzed following the procedure of Peterson (1977).

RESULTS

Morphology of trophosome tissue

Fresh trophosome from *R. pachyptila* is a gelatinous, pulpy, dark iridescent-green tissue. If the tissue is suspended in saline, numerous finger-like lobules project into the medium producing a villous appearance. Each lobule is approximately 0.15 mm in diameter and has a complex ultrastructure that is revealed by examining cross sections with electron microscopy (Fig. 1A).

The cells composing the outer pigmented layer of the lobule are tightly packed with membrane-bound inclusions of at least three morphological types. One type of granule (Fig. 1B, 2A) is homogeneous in composition, weakly electron-dense, and similar in appearance to mucus droplets or mucigen granules found in goblet cells of intestinal epithelia (e.g., Porter and Bonneville, 1973). In contrast, the darker osmophilic granules (Fig. 1B, 2B) contain highly organized, crystalline arrays of material (probably proteinaceous) that may be responsible for the intense green color of the trophosome. Indeed, if this outer cellular layer is osmotically lysed and removed from fresh trophosome tissue, the underlying tissue is white (R. Vetter, pers. comm.). The third type of inclusion (Fig. 1B) has an electron density intermediate to that of the previous two granules, and its appearance indicates a heterogeneous composition. Thus, based on the internal morphology, these pigmented cells comprising the outer layer of trophosome tissue are referred to hereafter as trophochrome cells.

Nuclei are visible in the trophochrome cells, but other common organelles (e.g., mitochondria, endoplasmic reticulum) are infrequent. The granules described above occupy the vast majority of the intracellular space. Even though it is possible that osmotic swelling could have accentuated the size of these inclusions, the structural integrity of both the limiting and internal cell membranes does not suggest extensive swelling.

Subtending the trophochrome cell layer are numerous bacterial endosymbionts (Fig. 3A, B). The bacteria housed in trophosome tissue are roughly spherical, often with irregular cell envelopes, and are approximately 3–5 microns in diameter. As one moves toward the center of the lobule, the morphology of the bacteria changes. Sulfur deposits within bacteria increase in both size and number, and ribosomes in the bacterial cytoplasm are less distinct compared to bacteria located toward the periphery of the lobule (Fig. 3A, B). (Note: sulfur deposits are identified as vacuoles where sulfur was extracted during tissue dehydration and embedding procedures.) Bacteria located toward the center of the lobule also appear smaller. Concentric membrane whorls within the bacteria are occasionally visible (Fig. 4A, upper left).

Although it is difficult to fully trace eucaryotic cell membranes, the following evidence indicates that the vacuole-enclosed bacteria are located within animal cells (bacteriocytes). There are nuclei, mitochondria, and other organelles interspersed among the bacteria (Fig. 4A, B). In Figure 4A, it is possible to delineate (moving

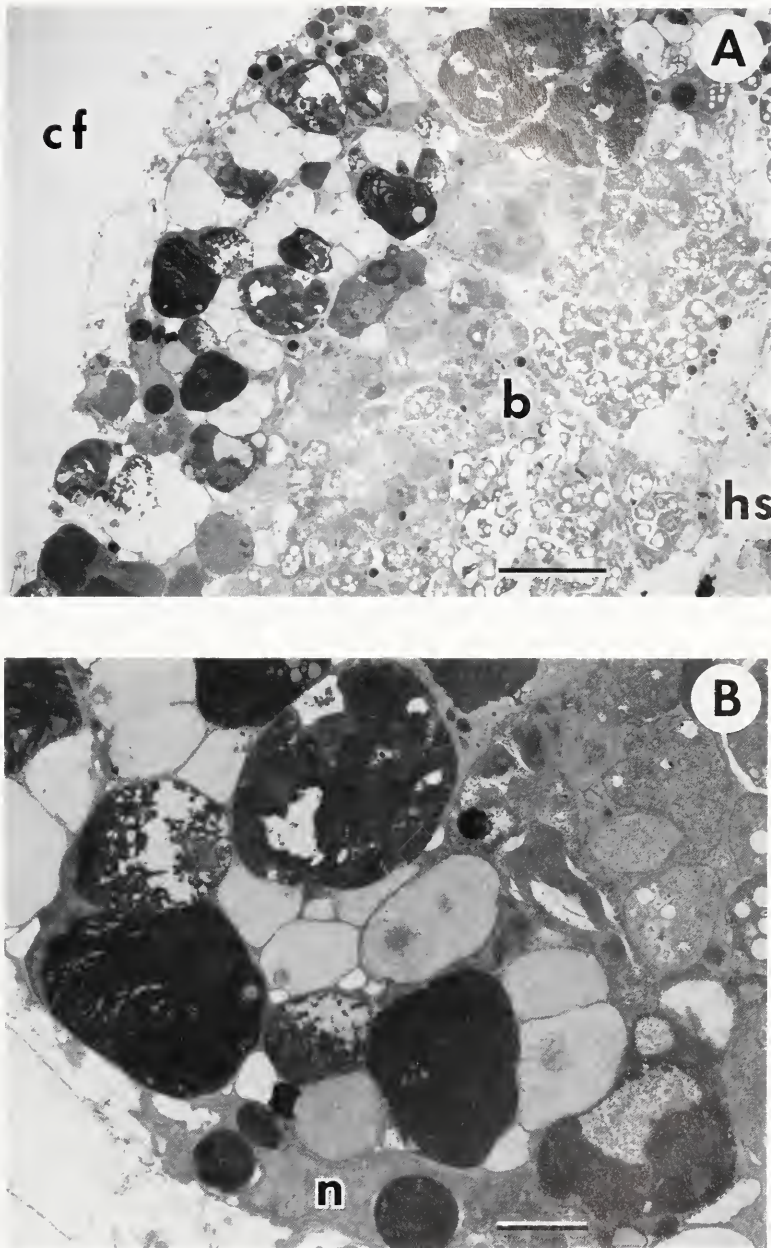


FIGURE 1. A. Cross section of a trophosome lobule from *Riftia pachyptila* viewed at low magnification with transmission EM. The outer trophochrome cell layer contacts the coelomic fluid space (cf). Subtending this pigmented cell layer are numerous bacteria (b). At the center of the lobule is a hemolymph space (hs). Scale bar = 10 microns. B. Higher magnification of the trophochrome cells showing a nucleus (n) and the tight intracellular packaging of diverse types of granules. Scale bar = 3 microns.

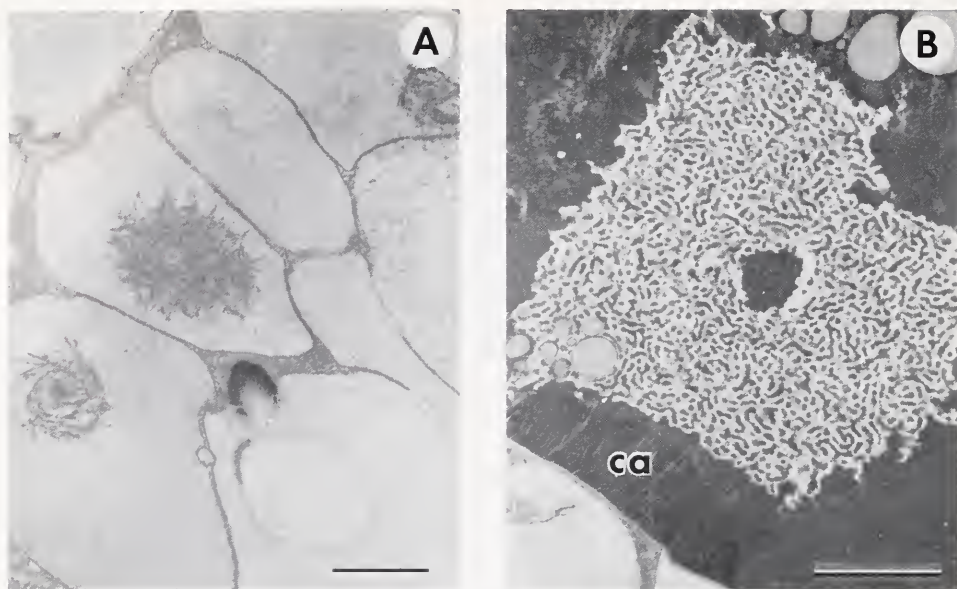


FIGURE 2. Two types of vacuole-enclosed granules present in trophochrome cells from *Riftia pachyptila*. A. Nondescript granules of uniform density, similar in appearance to mucigen granules. Scale bar = 1 micron. B. Electron-dense granule containing crystalline arrays (ca) of material that is proteinaceous in appearance. Scale bar = 2 microns.

outward from the center of the bacterium labeled "b") the bacterial cell envelope, the peribacterial membrane, and immediately adjacent, a nuclear envelope.

At the very center of the trophosome lobule, there is a hemolymph space or sinus extending longitudinally (Fig. 4B). We were unable to discern a basal lamina separating the bacteriocytes from the hemolymph space of the lobule.

Since the morphology and fine structure of the gill tissue from *C. magnifica* (Fiala-Medioni and Metivier, 1986) and *L. floridana* (Fisher and Hand, 1984) have already been described, we will not redescribe them here.

Isolated cell preparations

The cell purity for bacteriocytes isolated from bivalve gill tissue was approximately 70–80 percent (Table I). The amount of cellular debris present was not quantified, but was generally low. The yield of bacteriocytes was higher from *L. floridana* gill tissue than from *C. magnifica*. The reason may be that, while dissociation of *L. floridana* tissue was performed at 37°C, the incubation temperature for *C. magnifica* tissue had to be reduced to 20°C because of its temperature sensitivity. Although using 37°C incubations with the vent clam tissue improved the yield, the resulting bacteriocytes were not viable, as judged by the lack of ability to fix CO₂. Prior to the Percoll gradient step, the bacteriocytes represented 11% of the total cells in suspension from *L. floridana* gill. Thus, the density gradient fractionation achieved a 7-fold enrichment of bacteriocytes.

Attempts to isolate intact bacteriocytes from trophosome tissue were unsuccessful, suggesting that these cells are very fragile and are unable to withstand the isolation

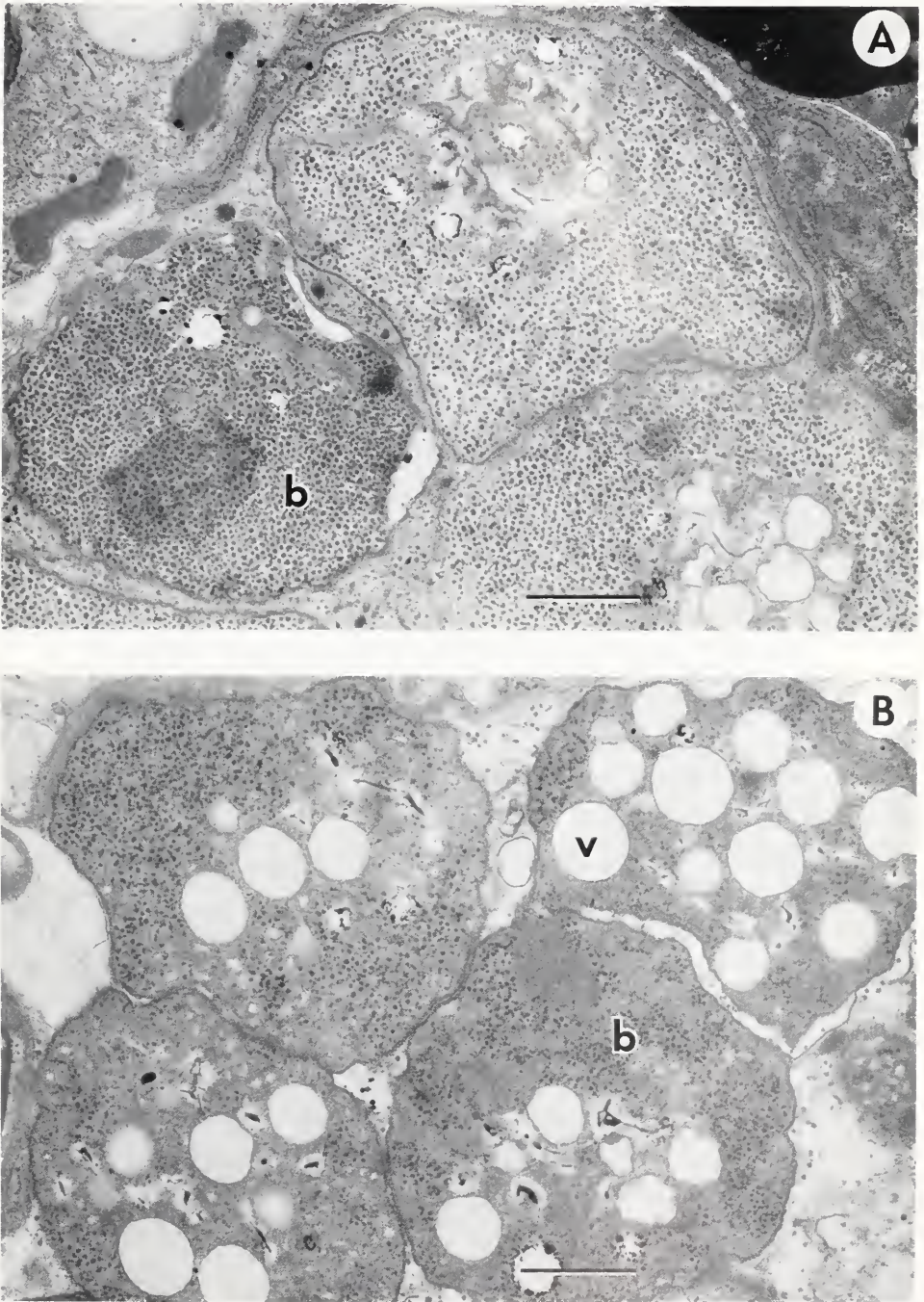


FIGURE 3. Bacteria (b) of *Riftia pachyptila* trophosome tissue. A. Located toward the periphery of the lobule, these bacteria are granular and contain very few sulfur deposits (observed as holes in the section where leaching has occurred). Scale bar = 1 micron. B. Bacteria located toward the center of the lobule contain more sulfur vacuoles (v), and their cytoplasm is less granular. Scale bar = 1 micron.

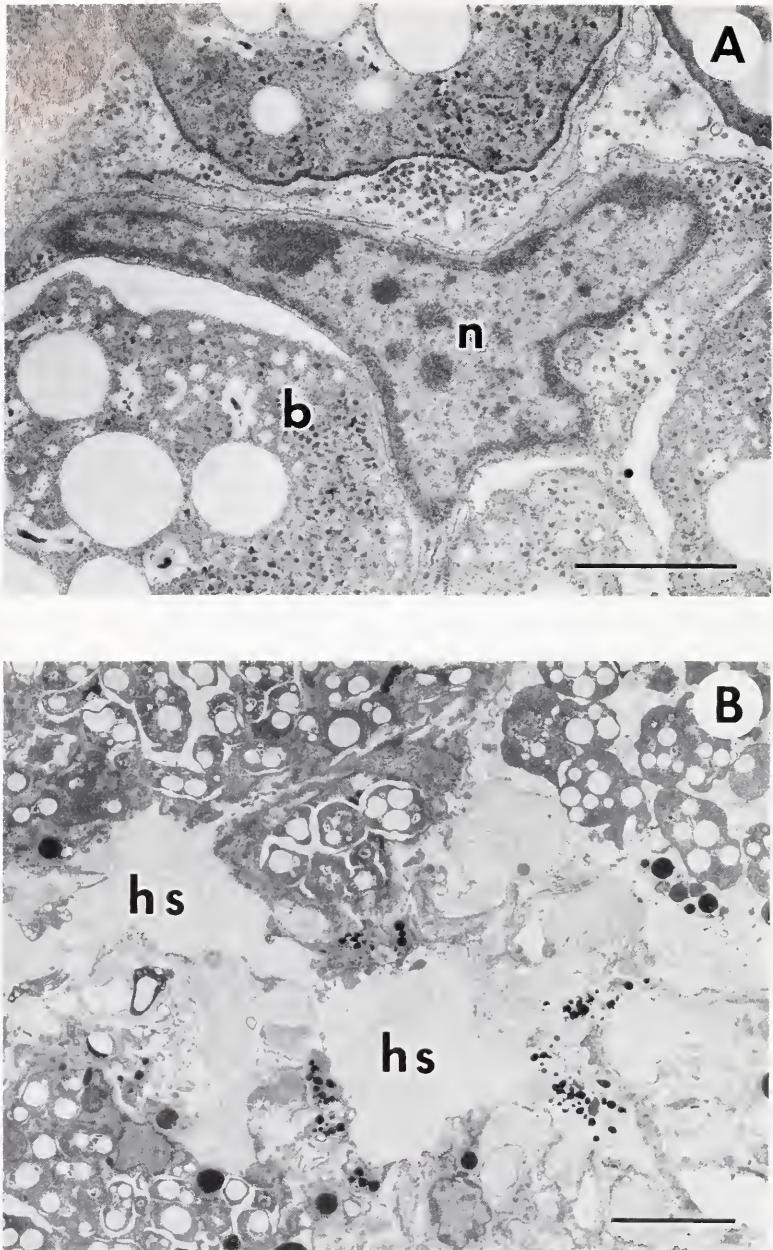


FIGURE 4. A. Bacterium (b) immediately adjacent to an animal cell nucleus (n). Scale bar = 1 micron. B. Micrograph showing hemolymph spaces (hs) located at the center of the lobule. Scale bar = 5 microns.

procedures. In contrast, trophochrome cells were enriched to a purity of 80%, and free bacteria were isolated in high yield and at a comparable purity to the pigmented trophosome cells. The primary contaminants of the trophochrome cell preparation were free bacteria.

TABLE I

Yield and purity of isolated cell preparations

Cell source and type	Gradient interface (% Percoll)	Yield (million cells/g tissue)	Purity (% of total)	Protein (mg/million cells)
<i>C. magnifica</i>				
Bacteriocytes	30–50%, 50–70%	1.16 ± 0.08 ^a (n = 3)	72 ± 5.6 (n = 5)	0.90 ± 0.14 (n = 3)
Non-bacteriocyte epithelial cells	10–30%	10.3	97	0.16
<i>L. floridana</i>				
Bacteriocytes	50–70%	3.0	81 ± 0.8 (n = 3)	—
<i>R. pachyptila</i>				
Bacteria	10–30%	—	79, 70	0.014
Trophochrome cells	30–50%, 50–70%	—	78, 82	1.09

^a Mean ± standard error.

One prominent ultrastructural feature of isolated bacteriocytes from *C. magnifica* is wide-spread fields of bacteria—easily the most numerous subcellular structures in the cytoplasm (Figs. 5A, C; 6A). Compared to the bacteria present in trophosome tissue, these bacteria are much smaller (0.5–0.7 micron diameter). They are clearly contained in vacuoles within the bacteriocyte, and nuclear regions are evident (Fig. 6A).

Large granules (approximately 5–10 micron diameter) are a second salient feature of bacteriocytes from both *C. magnifica* (Fig. 5A, C) and *L. floridana* (Fig. 5B). Some of the *C. magnifica* granules are irregularly shaped and quite electron-dense, and others are more circular and have a stippled appearance. The electron-dense granules are morphologically similar to those of bacteriocytes from *L. floridana* (Fig. 5B, and Fisher and Hand, 1984). When viewed with Nomarski differential-interference-contrast microscopy, the granules of *L. floridana* are striking and are certainly the dominant inclusion of the bacteriocyte (Fig. 5B). Previous work indicated the presence of iron in these granules (Fisher and Hand, 1984; cf., Wittenberg, 1985), but whether the bacteriocytes' granules in *C. magnifica* are chemically similar is unknown. As judged by the sedimentation behavior of bacteriocyte populations in Percoll gradients, *L. floridana* bacteriocytes have a greater density, which may be a consequence of differences between these pigment granules. Bacteriocytes from *L. floridana* also are over two-fold larger in diameter than those from *C. magnifica* (Fig. 5A, B).

The morphology of isolated trophochrome cells (Fig. 6B) is essentially unchanged from that seen for intact tissue (Fig. 1B). On the other hand, isolated bacteria look more irregular in overall shape compared to those viewed *in situ* and occasionally were more vacuolated, suggesting that the isolation procedure for this cell type needs improvement.

Metabolic properties of isolated cells

The capacity for carbon dioxide fixation of *C. magnifica* bacteriocytes and isolated bacteria from *Riftia* trophosome is presented in Table II. In the absence of sulfur compounds, incorporation of CO₂ by bacteriocytes proceeds at a rate of 13 nmoles CO₂/mg protein/h. Fixation is inhibited by sulfide and, to a lesser degree, thiosulfate

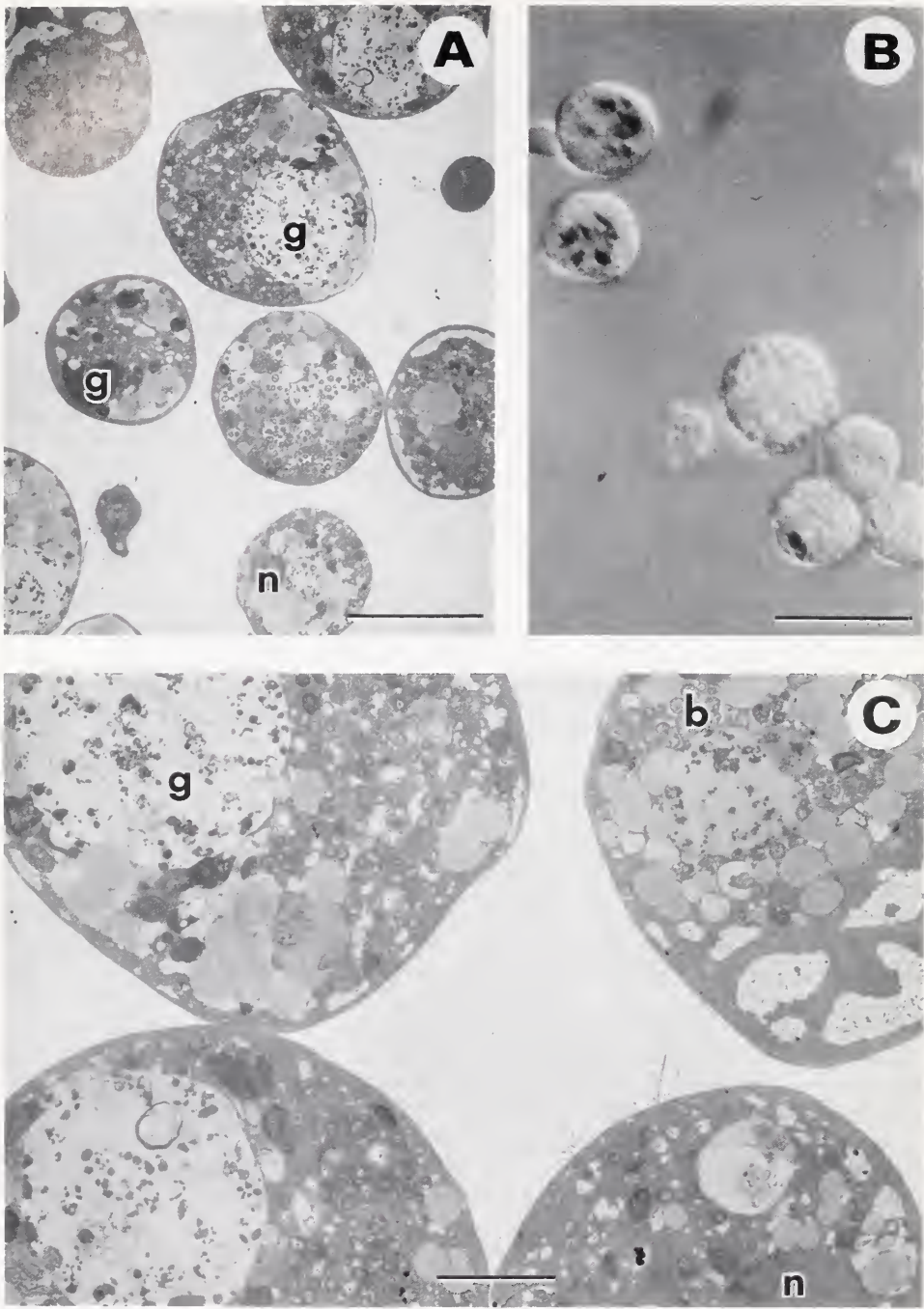


FIGURE 5. A. Isolated bacteriocytes from the gill tissue of *Calyptogenia magnifica* viewed with transmission EM. Two distinctly different granules (g) are observable in the cytoplasm. Scale bar = 15 microns. B. Nomarski light micrograph of bacteriocytes from *Lucina floridana* gill tissue, emphasizing the prominent granules in these very large cells. Scale bar = 60 microns. C. Higher magnification of *C. magnifica* bacteriocytes illustrating the expansive fields of symbiotic bacteria (b). Nucleus (n), granules (g). Scale bar = 5 microns.

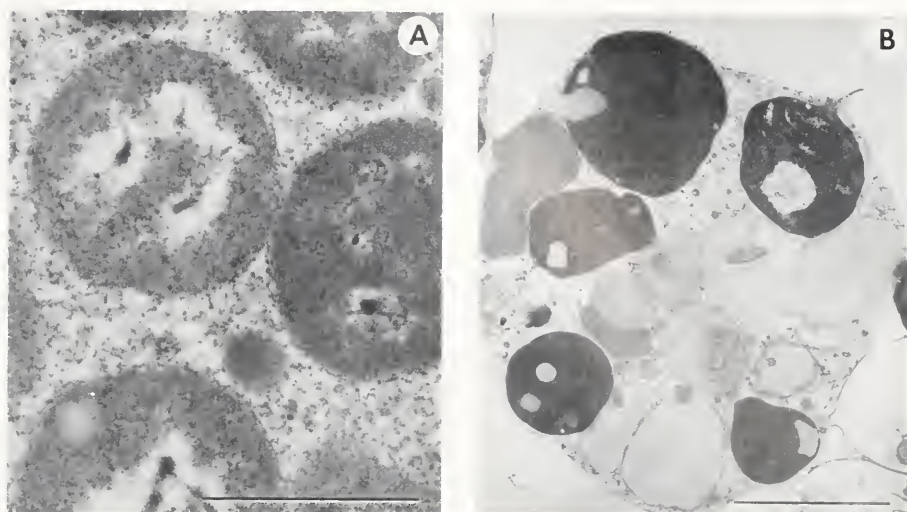


FIGURE 6. A. High magnification (78,000 $\times$) of the symbiotic bacteria present in the cytoplasm of isolated bacteriocytes from *Calyptogenia magnifica*. Note the peribacterial membrane encompassing each bacterium, and the distinct nuclear regions. The bacteria are approximately an order of magnitude smaller than those in *Riftia* trophosome. Scale bar = 0.5 micron. B. Isolated trophochrome cell from *Riftia pachyptila*. Scale bar = 5 microns.

across the range of concentrations used here. The rate of fixation by *L. floridana* bacteriocytes is approximately doubled by the addition of 0.1 mM thiosulfate; higher thiosulfate concentrations do not appreciably alter the fixation rate. Incorporation by non-bacteriocyte epithelial cells of *C. magnifica* is approximately one third the rate of *C. magnifica* bacteriocytes. The incorporation by the non-bacteriocyte preparation is presumably due to eucaryotic enzymes like pyruvate carboxylase and phosphoenolpyruvate carboxykinase, both known to occur in marine bivalves (e.g., Felbeck, 1983; Meinardus-Hagar and Gäde, 1986). Contamination by bacteriocytes in this preparation is low (Table I).

The positive influence of sulfide on the intracellular ATP levels of *C. magnifica* bacteriocytes contrasts with the inhibitory effect seen on CO_2 fixation (Table III). After 60 min, bacteriocytes incubated in 0.5 mM sulfide contain 3.30 nmoles ATP/million cells (approximately 1.01 mM), compared to 1.02 nmoles/million cells (0.31 mM) with no added sulfur, and 1.74 nmoles/million cells (0.54 mM) with 0.5 mM thiosulfate.

DISCUSSION

The primary objectives of this study were (1) to describe ultrastructural features of *Riftia* trophosome tissue, particularly those related to the distribution and subcellular location of bacteria and the nature of the pigmented layer of trophochrome cells, (2) to prepare suspensions of isolated cells from invertebrate tissues that contain bacterial endosymbionts, and (3) to use these cellular preparations for characterizing metabolic aspects of the symbioses.

Morphological evidence supports the conclusion that symbiotic bacteria present in the trophosome of *R. pachyptila* are contained within animal cells (i.e., bacterio-

TABLE II

Incorporation of carbon dioxide in isolated cell suspensions at 20°C

Preparation	Sulfur compound present	CPM/million cells/h	nmoles CO ₂ /million cells/h	nmoles CO ₂ /mg protein/h	
<i>C. magnifica</i>					
Bacteriocytes	none	5632	13.9	13.0	
		5976	14.8	13.3	
	Na ₂ S: 0.1 mM	4590	11.4	—	
		4272	10.6	9.5	
	0.5 mM	3696	9.1	—	
		4224	10.5	9.5	
	1.0 mM	2676	6.6	—	
	Na ₂ S ₂ O ₃ : 0.1 mM	4488	11.1	—	
		5904	14.6	13.2	
	0.5 mM	3084	7.6		
		3684	9.1	8.1	
	1.0 mM	3792	9.4	8.4	
	Non-bacteriocyte epithelial cells	none	292	0.72	4.5
		Na ₂ S: 0.1 mM	404	0.10	6.2
0.5 mM		302	0.75	4.6	
1.0 mM		318	0.79	4.8	
1.5 mM		348	0.86	5.3	
2.0 mM		136	0.34	2.1	
Na ₂ S ₂ O ₃ : 0.1 mM		292	0.72	4.5	
0.5 mM		266	0.66	4.0	
1.0 mM		246	0.61	3.8	
1.5 mM		274	0.68	4.2	
2.0 mM		292	0.72	2.2	
<i>L. floridana</i>					
Bacteriocytes		none	3440	8.4	—
		Na ₂ S ₂ O ₃ : 0.1 mM	7776	19.0	—
	0.2 mM	7176	17.6	—	
	0.5 mM	8728	21.4	—	
	0.75 mM	7912	19.4	—	
	1.0 mM	8824	21.6	—	
<i>R. pachyptila</i>					
free bacteria	none	—	—	13.5	

cytes). Electron micrographs show that nuclei and other eucaryotic organelles are frequently interspersed among the vacuole-enclosed bacteria, and eucaryotic cell membranes appear to enclose bacteria and such organelles within the same cell. Cavanaugh (1985) suggested that an intracellular location for the bacteria was probable, but previous evidence has been inconclusive (Cavanaugh *et al.*, 1981; Cavanaugh 1983, 1985).

A second notable point regarding trophosome fine structure is the distribution of sulfur deposits among the bacteria within a trophosome lobule. The bacteria located closer to the outer trophochrome layer clearly have fewer deposits than do bacteria located nearer the hemolymph space at the center of the lobule. This spatial distribution could reflect greater access of the latter bacteria to high sulfide concentrations in

TABLE III

Intracellular ATP levels of Calyptogenia magnifica bacteriocytes in the presence of sulfur compounds at 20°C

Incubation time ¹ (min)	Sulfur compound	nmoles ATP/ million cells	Calculated intracellular ATP concentration (mM) ²
0	none	1.34	0.41
30	none	0.83	0.25
	0.5 mM Na ₂ S	1.34	0.41
	0.5 mM Na ₂ S ₂ O ₃	0.88	0.28
60	none	1.02	0.31
	0.5 mM Na ₂ S	3.30	1.01
	0.5 mM Na ₂ S ₂ O ₃	1.74	0.54
90	none	2.03	0.62
	0.5 mM Na ₂ S	3.75	1.15
	0.5 mM Na ₂ S ₂ O ₃	1.29	0.40

¹ Cells were incubated at 20°C in artificial seawater (pH 8.2) containing the indicated concentrations of sulfide and thiosulfate.

² The intracellular concentrations of ATP were calculated using an average bacteriocyte diameter of 19.8 ± 0.8 microns (SE, $n = 25$), as determined from transmission electron micrographs. Cellular water content was assumed to be 80%.

Riftia vascular blood (Childress *et al.*, 1984). Indeed, Vetter (1985) showed that in the absence of sulfide, sulfur globules are lost from bacterial symbionts in the gill tissue of *Lucinoma annulata*; presumably the presence of sulfide would stimulate deposition of these elemental sulfur stores. The occurrence and possible roles of sulfur deposits in free-living sulfur bacteria recently have been reviewed (Vetter, 1985). It should be noted that the coelomic fluid of *Riftia* also contains sulfide, but it is unclear whether this sulfide pool is available directly to the symbionts, or must first be transferred to the vascular circulation (Childress *et al.*, 1984). If the density of sulfur deposits is related to the level of sulfur-based metabolism occurring in a given bacterium, then a gradient of metabolic activity could exist, with higher metabolic potential being possessed by bacteria closer to the central hemolymph space of the lobule. Bacteria near the hemolymph space also appear smaller, which is consistent with them being younger than the larger bacteria toward the outer trophochrome layer.

The morphology of the pigmented layer of trophosome tissue was far more complex than originally anticipated. First, the trophochrome layer does not contain bacterial endosymbionts, although the cells do contain extensive numbers of cytoplasmic inclusions (Figs. 1B, 2A, B). The function(s) of these cells is not understood presently, but their internal structure suggests several possibilities. Some of the intracellular droplets or granules are structurally similar to those found in mucus-secreting cells (*e.g.*, intestinal goblet cells). What benefit the secretion of mucus-like material (if it were to occur) by trophochrome cells would confer to the tubeworm is unclear, other than possibly lubricating the external surface of the trophosome that is in direct apposition to the internal surface of the body wall of the worm. Another possible function of trophochrome cells, that of phagocytosis and degradation of aging bacteria, is suggested by the heterogeneous contents of other intracellular granules (Fig. 1B). Future experiments measuring levels of protease activity in isolated trophochrome cells could be enlightening in this context. Finally, trophochrome cells also contain electron-dense material that is deposited as crystalline arrays in some granules (Fig. 4B).

This material is likely proteinaceous and may be the pigment responsible for the deep green color of the trophosome.

The region of the trophosome containing the bacteriocytes is a diffuse, loosely associated tissue, which suggests that its dissociation into suspensions of single bacteriocytes should have been relatively straight forward. Apparently however, the cells are very fragile, and we were unable to obtain intact bacteriocytes from trophosome even when mechanical agitation was kept to a minimum.

Thus, the isolated cellular preparation that may have the most potential for improving our understanding of the mechanisms involved in metabolic utilization of sulfur by the symbiosis is the bacteriocytes from *C. magnifica* and *L. floridana*. The ultrastructure of these isolated cells is comparable to the structure as viewed *in situ* (Fisher and Hand, 1984; Fiala-Medioni and Metivier, 1986), with the exception that bacteria in isolated bacteriocytes from *C. magnifica* appear a little more spherical than those in intact tissue (pers. obs., and Fiala-Medioni and Metivier, 1986). The size of *C. magnifica* bacteria differs markedly from those of *Riftia* trophosome, and data from 5S rRNA sequencing suggest the bacteria from the two sources have minimal affiliation (Stahl *et al.*, 1984; Lane *et al.*, 1985).

The temperature sensitivity of *C. magnifica* bacteriocytes closely parallels the observations of Belkin *et al.* (1986) in their studies with gill homogenates of the vent mussel *Bathymodiolus thermophilus*. In both cases, the capacity for CO₂ fixation declines dramatically at temperatures above 20°C. Thus, the bacterial endosymbionts from *C. magnifica* are comparable to those of *B. thermophilus* in that both appear to be sulfur-oxidizing bacteria with psychrophilic characteristics (*cf.*, Belkin *et al.*, 1986). The detrimental effect of warm temperature was not observed for bacteriocytes from the shallow-water bivalve *L. floridana*, and in general this cellular preparation seemed harder.

In the absence of an exogenously added sulfur source, the rate of CO₂ fixation by *C. magnifica* bacteriocytes was 13 nmoles/mg protein/h. Rates obtained with homogenates of *B. thermophilus* gill ranged between 1.7 and 9.4 nmoles/mg protein/h, depending on temperature and the thiosulfate concentrations present (Belkin *et al.*, 1986). Using "purified" bacteria from *B. thermophilus*, Belkin *et al.*, (1986) reported maximum CO₂ fixation rates of approximately 40 nmoles/mg protein/h in the presence of thiosulfate. In the present study, CO₂ fixation by *C. magnifica* bacteriocytes was inhibited by sulfide across the range of 0.1 to 1.0 mM. Less inhibition was seen with thiosulfate. Similarly, Anderson (1986) showed that CO₂ fixation by whole specimens of *Solemya reidi* was inhibited at sulfide concentrations above 0.1 mM in surrounding seawater. This protobranch bivalve has a chemoautotrophic metabolism based on the presence of sulfur bacteria localized in its gill tissue (Felbeck, 1983). Unfortunately, we do not have measurements of CO₂ fixation by bacteriocytes at sulfide concentrations below 0.1 mM. Considering Anderson's results (1986), such sulfide levels could well stimulate the process.

CO₂ fixation by *L. floridana* bacteriocytes was increased approximately two-fold by thiosulfate concentrations between 0.1 and 1.0 mM, a pattern virtually identical to that seen for gill homogenates of *B. thermophilus* (Belkin *et al.*, 1986). Exogenously added sulfide was minimally effective in stimulating the process in *L. floridana* bacteriocytes (preliminary observations) and in *B. thermophilus* homogenates (Belkin *et al.*, 1986). The rate of CO₂ fixation by isolated bacteria from *R. pachyptila* trophosome was 13.5 nmoles/mg protein/h in the absence of added sulfur. The response to sulfide and thiosulfate was highly variable, and thus the results are not reported here. This variability could be related to the general instability of the CO₂ fixation capacity exhibited by trophosome preparations (Belkin *et al.*, 1986).

Although sulfide (0.1 mM and above) inhibited CO₂ fixation by isolated bacteriocytes from *C. magnifica*, the presence of 0.5 mM sulfide promoted higher intracellular ATP concentrations than control incubations without any added sulfur. The opposing effects of sulfide on the two processes may be related to the sulfide concentrations used; as discussed earlier, if lower sulfide levels had been tried, CO₂ fixation might also have been stimulated. Thiosulfate was not as effective in enhancing intracellular ATP levels. Powell and Somero (1986a), using lysed bacterial preparations from *C. magnifica* gill, showed that sulfite stimulated ATP synthesis while thiosulfate and sulfide did not. In their study, ATP was measured using the firefly luciferase technique, and incubations were of shorter duration. Most likely, components of the animal cell contributed to sulfide utilization in our study with isolated bacteriocytes. Although some degree of spontaneous oxidation is possible, Powell and Somero (1985) localized sulfide oxidizing bodies in the animal cell cytoplasm of *S. reidi* gill tissue. These workers also have conclusively demonstrated that mitochondria isolated from *Solemya reidi* gill tissue can couple sulfide oxidation to the formation of ATP via the electron transport system and oxidative phosphorylation (Powell and Somero, 1986b).

Thus, the initial step(s) of sulfide oxidation could occur in bacteriocytes prior to the sulfur compound reaching the endosymbiont, and some of the ATP synthesis in bacteriocytes could reflect mitochondrial processing. Consequently, the identities of sulfur compounds exploited for chemical energy by the symbiosis may depend on the structural organization and integrity of the biological preparation under study. Additional metabolic characteristics of invertebrate-sulfur bacteria symbioses may display similar dependencies in ways yet to be identified.

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ENERGETICS OF CONTRACTILE ACTIVITY IN ISOLATED RADULA PROTRACTOR MUSCLES OF THE WHELK *BUSYCON CONTRARIUM*: ANAEROBIC END PRODUCT ACCUMULATION AND RELEASE

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ABSTRACT

Anaerobic energy metabolism during contractile activity was investigated in the isolated radula protractor muscle of the whelk *Busycon contrarium*. Spectrophotometric assay of enzyme activities in crude tissue extracts revealed particularly high pyruvate reductase activities with octopine dehydrogenase displaying the highest activity. During electrically induced isotonic contractions of the radula protractor muscles, the following end products, listed in order of increasing level, accumulated in the tissue: strombine, octopine and alanopine (the "opines"), and D-lactate. Pyruvate levels increased three-fold during muscle contraction, suggesting that pyruvate plays a key role in the regulation of the pyruvate reductases. The muscle released lactate, but none of the opines, into the incubation medium, with rates exceeding $3 \mu\text{moles} \cdot \text{min}^{-1} \cdot \text{g wet wt}^{-1}$. During the later phases of contraction, more D-lactate was released into the medium than accumulated in the muscle. We conclude that transport of D-lactate permits sustained flux through lactate dehydrogenase because of a reduction in product inhibition. Furthermore, we hypothesize that D-lactate transport may be coupled to H^+ export or OH^- import, which would then serve to regulate the extent of accumulation of glycolytically produced protons.

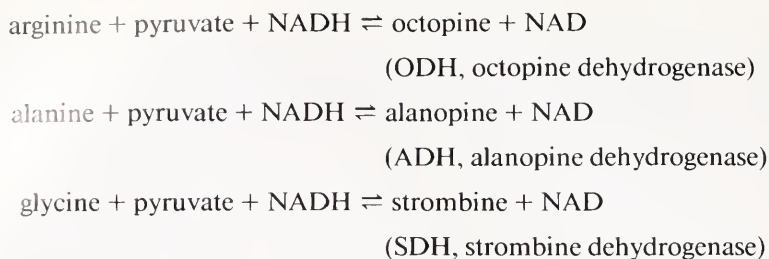
INTRODUCTION

The muscles of marine molluscs possess two distinctly different mechanisms of energy production during periods of reduced oxygen availability. During environmental anaerobiosis—*i.e.*, whole-organism exposure to anoxia—aspartate and glycogen are cofermented, yielding succinate and alanine as end products (Gäde, 1983). The succinate pathway typically occurs at relatively low rates and is associated with a reverse Pasteur effect in these muscles (Storey, 1985). A number of molluscs may undergo functional anaerobiosis, where oxygen demand exceeds delivery. In this instance, only certain tissues are rendered anoxic (Gäde, 1983). Under these conditions, the glycolytic flux is several orders of magnitude higher than under conditions of environmental anaerobiosis (Livingstone, 1982; Gäde, 1983). The higher energy outputs necessary for burst activity are provided by glycogen fermentation and the shunting of pyruvate through pyruvate reductases such as lactate and opine dehydrogenases (Gäde and Grieshaber, 1986), resulting in the accumulation of D-lactate, octopine, alanopine, or strombine.

Opine dehydrogenases catalyze the reductive condensation of pyruvate and an amino acid according to the following general reactions:

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Many molluscan muscles have the enzymatic potential for producing several opine end products as well as D-lactate since high activities of opine and D-lactate dehydrogenases may occur in the same tissue (Zammit and Newsholme, 1976; Livingstone *et al.*, 1983). For example, the pedal retractor (Baldwin *et al.*, 1981; Baldwin and England, 1982) and radula (Ellington, 1982) muscles of gastropods contain significant activities of all opine dehydrogenases as well as D-lactate dehydrogenase. The relative contribution of these enzymes to the maintenance of glycolytic flux during contractile activity, not yet fully explored, is considered in this paper.

The metabolic disposition of end products of anaerobic metabolism in molluscs (Ellington, 1983b) is poorly known. Propionate is released into the hemolymph of *Mytilus edulis* (Zurburg *et al.*, 1982), whereas succinate appears to accumulate in the hemolymph of the clam *Mercenaria mercenaria* (Korycan and Storey, 1983). Octopine is not released into the hemolymph during contractile activity in the giant scallop, *Placopecten magellicanus* (de Zwaan *et al.*, 1980). However, hemolymph octopine levels are slightly elevated after contractile activity or hypoxia in the cephalopods *Sepia officinalis* and *Loligo vulgaris* (Storey and Storey, 1979; Gäde, 1980). Alanopine and strombine appear not to be released from molluscan muscles. Octopine, alanopine, and strombine levels fall during recovery, indicating oxidation *in situ* (Ellington, 1983b).

The present study focuses on the metabolism of the radula protractor muscle of the large marine gastropod *Busycon contrarium*. This muscle possesses relatively high activities of lactate and opine dehydrogenases. The presence of several pyruvate reductases in the radula protractor muscle poses questions about the control of these enzymes and the disposition of their products. We show that electrical stimulation of this muscle while it is subjected to anoxia induces the formation of all opines as well as D-lactate. Interestingly, formation of D-lactate is much greater than that of the other end products, even though the activities of the opine dehydrogenases are much higher. Further, D-lactate is released from the exercising muscle into the medium while the opines are retained. End-product removal may enhance the formation of additional D-lactate and, as a result, large amounts of carbon can be shunted through lactate dehydrogenase allowing for higher, sustained glycolytic fluxes during anoxia.

MATERIALS AND METHODS

Animals

Specimens of the whelk *Busycon contrarium* were collected off Alligator Point in Franklin County, Florida, and were maintained in the flowing-seawater system at the Florida State University Marine Laboratory near St. Theresa. Individuals used in experiments were transferred to the Florida State University campus, where they were maintained for brief periods in a recirculating seawater system.

Biochemicals

Biochemicals were purchased from Boehringer-Mannheim (Indianapolis) and Sigma Chemical Company (St. Louis). D-Lactate dehydrogenase, used to determine D-lactate, was purified from the muscle of the horseshoe crab *Limulus polyphemus*. Octopine dehydrogenase, used to assay for octopine, arginine, and arginine phosphate, was purified from the adductor muscles of the scallop *Argopecten irradians concentricus*. Succinyl Co A synthase, used in succinate assays, was a gift from Dr. William Bridger, Department of Biochemistry, University of Alberta, Edmonton.

Experimental procedure

Intact radula protractor muscles, dissected from the proboscis apparatus, were ligated at both ends with surgical silk and placed in a 5×75 -mm muscle bath filled with 1.5 ml of MBL (Marine Biological Laboratory) formula artificial seawater buffered with 5 mM hydroxyethylpiperazine ethanesulfonic acid (pH = 7.8). One end of the muscle was fastened to a hook electrode and pulled into a rubber sleeve at the bottom of the muscle bath. The other end was attached to a Narco Biosystems isotonic myograph transducer with the silk suture. Muscles were suspended in the bath at 1.5 times their resting length (measured upon excision). Temperature was maintained by immersion of the bath in a larger water-filled vessel which was jacketed and controlled by a Brinkmann model RM 6 recirculating water bath (20°C). Contractions were recorded with the isotonic transducer connected to a Narco Biosystems model MK IV physiograph. A second electrode, inserted in the bath, delivered square-wave pulses (60 volts, 40 ms) at 2.5-s intervals from a Grass model SD9 stimulator. The bath was gassed with normocapnic nitrogen (0.05% CO₂) through a 75-mm, 22-gauge Luer lock syringe needle.

Each muscle preparation was fastened in the bath and bubbled with nitrogen for 15 min. Control experiments were terminated at this point, and the tissues were removed, blotted, and frozen in liquid nitrogen. Experimental groups were gassed as the controls were, and then subjected to various periods of electrical stimulation (2.5, 5, 10, and 15 min) in the presence of normocapnic nitrogen before being frozen. In all cases, the medium was decanted from the bath and stored at -70°C for later analysis.

Enzyme assays

Freshly dissected muscles were homogenized in 24 volumes of extraction buffer (50 mM triethanolamine, pH 7.4, 1 mM EDTA, 20 mM mercaptoethanol, 20% glycerol) with a Tekmar UltraTurrax tissue homogenizer. The homogenate was centrifuged at $12,000 \times g$ for 20 min at 4°C. The supernatant was passed through a Sephadex G-25 column (1.5 $\times$ 14 cm) equilibrated with extraction buffer less glycerol, which removed low-molecular-weight compounds. The proteins in the void volume were used as the source of enzyme activities. The activities were determined spectrophotometrically with a Gilford model 252-1 spectrophotometer according to the methods outlined by Ellington (1982).

Metabolite assays

Nutralized perchloric acid extracts were prepared from the radula protractor muscles frozen at -70°C according to the methods of Graham and Ellington (1985). Arginine phosphate was assayed spectrophotometrically by the method of Grieshaber

TABLE I

Profile of pyruvate reductase activities in desalted tissue extracts of radula protractor muscles from *Busycon contrarium*

Enzyme	Activity
D-Lactate dehydrogenase	36.46 $\pm$ 9.71
Strombine dehydrogenase	61.98 $\pm$ 14.88
Alanopine dehydrogenase	96.87 $\pm$ 19.66
Octopine dehydrogenase	509.83 $\pm$ 40.73

Activities are expressed in $\mu\text{moles} \cdot \text{min}^{-1} \text{g wet wt}^{-1}$ and were measured at 25°C. Data represents mean $\pm$ 1 SD, n = 4.

and Gäde (1976). Pyruvate was assayed fluorometrically in a Farrand Optical model A-4 fluorometer by the method of Lowry and Passonneau (1972). Both pyruvate and arginine phosphate levels were determined immediately after neutralization to eliminate sample loss.

Aspartate, succinate, arginine, and malate were determined spectrophotometrically according to the method of Williamson and Corkey (1969), Williamson (1974), Grieshaber and Gäde (1976), and Williamson and Corkey (1969), respectively. Octopine and lactate were assayed fluorometrically essentially as outlined by Graham and Ellington (1985).

Concentrations of the free amino acids alanine and glycine were determined by HPLC on a Dionex amino acid analyzer with a Pierce amino acid column and buffers (Pierce Chemical Company). Alanopine and strombine concentrations were also determined by HPLC methods (Fiore *et al.*, 1984).

RESULTS

Enzyme activities

Freshly prepared extracts of the radula protractor muscles of *Busycon contrarium* displayed high activities of all four pyruvate reductases (Table I). Octopine dehydrogenase (ODH) had the highest activity of the enzymes assayed. Alanopine dehydrogenase (ADH) displayed somewhat lower activity followed by strombine (SDH) and D-lactate (LDH) dehydrogenases (Table I).

Contractile activity

Contractile activity under nearly anoxic conditions was maintained within 98% of initial values for the first 5 min of electrical stimulation (Fig. 1). There was a general trend towards a decline in force thereafter. This pattern was evident in all muscle preparations tested.

Metabolite levels in the tissue and the medium

Arginine phosphate levels declined at the onset of muscular activity, reached a minimum after 10 min, and remained relatively constant thereafter (Table II). Free arginine levels increased in the first 2.5 min of contractile activity, then fell to near control levels at the end of the experiment (Table II). Within 10 min, alanine levels were three times greater than levels measured initially, but returned to near control levels after 15 min (Fig. 2). Glycine levels did not change significantly during the 15

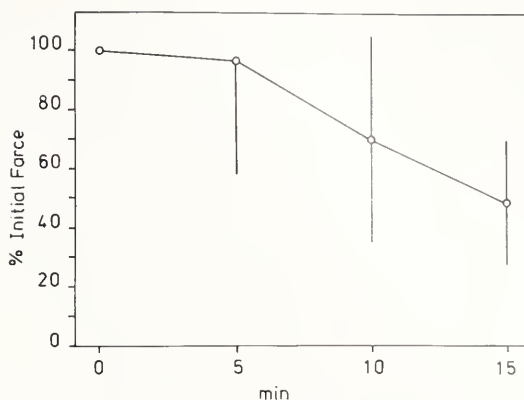


FIGURE 1. Changes in contractile force for electrically stimulated isotonic contractions of isolated radula protractor muscles from *Busycon contrarium* versus time. Units are percent change of initial force. Points represent means $\pm$ 1 SD, $n = 5$.

min of contractile activity (Fig. 2). Aspartate levels in the muscle declined during contractile activity, with the bulk of the change taking place in the first 5 min (Fig. 2). Succinate and malate levels in muscles stimulated for 15 min were less than $0.5 \mu\text{moles} \cdot \text{g wet wt}^{-1}$.

After 2.5 min of stimulation, pyruvate levels in the muscle increased dramatically (Fig. 3). Strombine was not a major glycolytic end product, as this compound accumulated to levels approaching only $1 \mu\text{mole} \cdot \text{g wet wt}^{-1}$ (Fig. 3). In contrast, octopine accumulated linearly during the experiment (Fig. 3, Table II). The sum of octopine, free arginine, and arginine phosphate levels was constant (Table II), indicating no net change in the total arginine pool in the tissue. Alanopine showed the highest accumulation of all the opines, but formation did not begin immediately as this compound was not detectable in the first 2.5 min of stimulation (Fig. 3). D-lactate was the predominant end product formed in the muscle, exhibiting a dramatic increase during the later periods of contraction (Fig. 3).

Octopine, alanopine, and strombine were not released into the medium by the muscle preparations. In contrast, a significant amount of D-lactate was found in the

TABLE II

Total arginine pool (arginine phosphate, arginine, octopine, and total arginine) for neutralized perchloric-acid extracts of isotonically contracting radula protractor muscles isolated from *Busycon contrarium*

Time (min)	Metabolite			
	Free arginine	Arginine phosphate	Octopine	Total
0	2.37 ± 2.41	10.69 ± 3.77	$0.46 \pm .29$	13.47 ± 4.39
2.5	4.44 ± 1.17	7.35 ± 2.83	$1.11 \pm .85$	12.55 ± 3.34
5	3.75 ± 1.04	6.98 ± 1.50	$1.71 \pm .38$	12.44 ± 1.18
10	$2.71 \pm .83$	4.93 ± 2.75	3.47 ± 1.64	10.76 ± 2.84
15	$2.44 \pm .48$	6.98 ± 2.64	4.72 ± 1.73	14.14 ± 1.26

Levels are expressed in $\mu\text{moles} \cdot \text{g wet wt}^{-1}$. Data represent means $\pm$ 1 SD, $n = 5$.

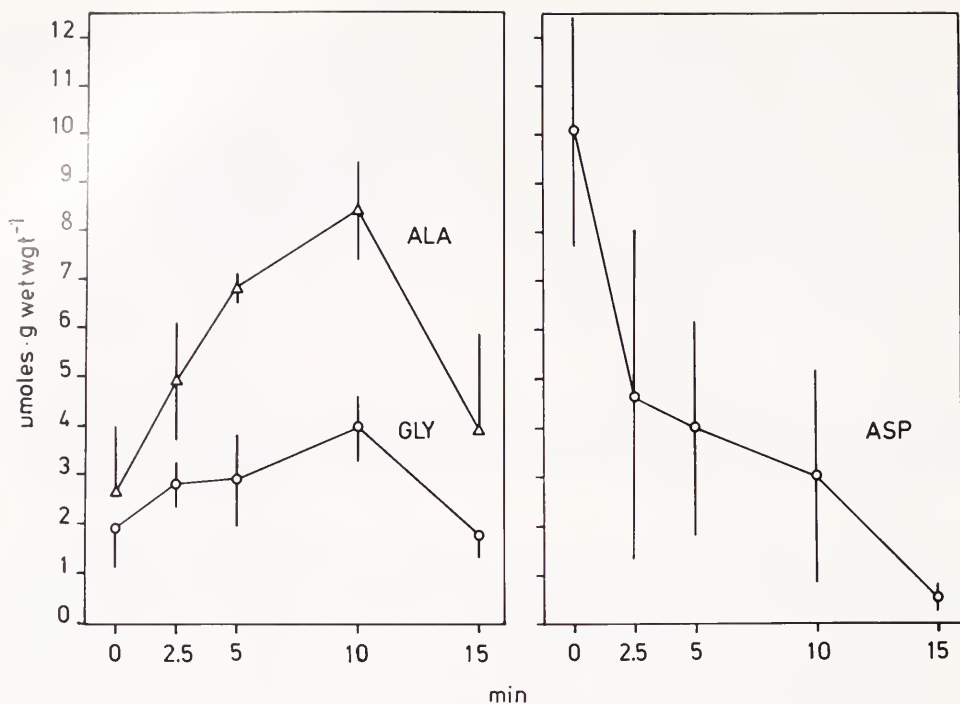


FIGURE 2. Changes in levels of the amino acids alanine (ALA), glycine (GLY), and aspartate (ASP) as determined by HPLC of neutralized perchloric-acid extracts of electrically stimulated radula protractor muscles from *Busycon contrarium* over time. Units are $\mu\text{moles} \cdot \text{g wet wt}^{-1}$. Points represent means $\pm$ 1 SD, n = 5.

medium (Fig. 4). In fact, during the last 5 min, more D-lactate was released into the medium than accumulated in the muscle (Fig. 4).

DISCUSSION

The high activities of all four pyruvate reductases in the radula protractor muscle of *B. contrarium* are similar to those observed in the radula retractor muscles of this species (Ellington, 1982) and in other gastropod muscles (Baldwin and England, 1982; Livingstone *et al.*, 1983). The highest activity, exhibited by octopine dehydrogenase, was comparable to activities observed in cephalopod molluscs (Baldwin and England, 1980). The simultaneous accumulation of D-lactate, alanopine, octopine, and to a lesser extent strombine, which occurs in the radula protractor system during muscular activity, indicates that all four of these reductases operate under functional anoxia. The accumulation of these end products is temporally correlated with changes in pyruvate levels. Pyruvate levels increased dramatically during the time course of muscle contraction.

Pyruvate reductases are thought to be equilibrium enzymes (de Zwaan and Dando, 1984; Gäde and Grieshaber, 1986) and are thus regulated by changes in the concentrations of substrates and products. Pyruvate is the common substrate for all four reductases. The observed elevations in pyruvate levels in *B. contrarium* radula

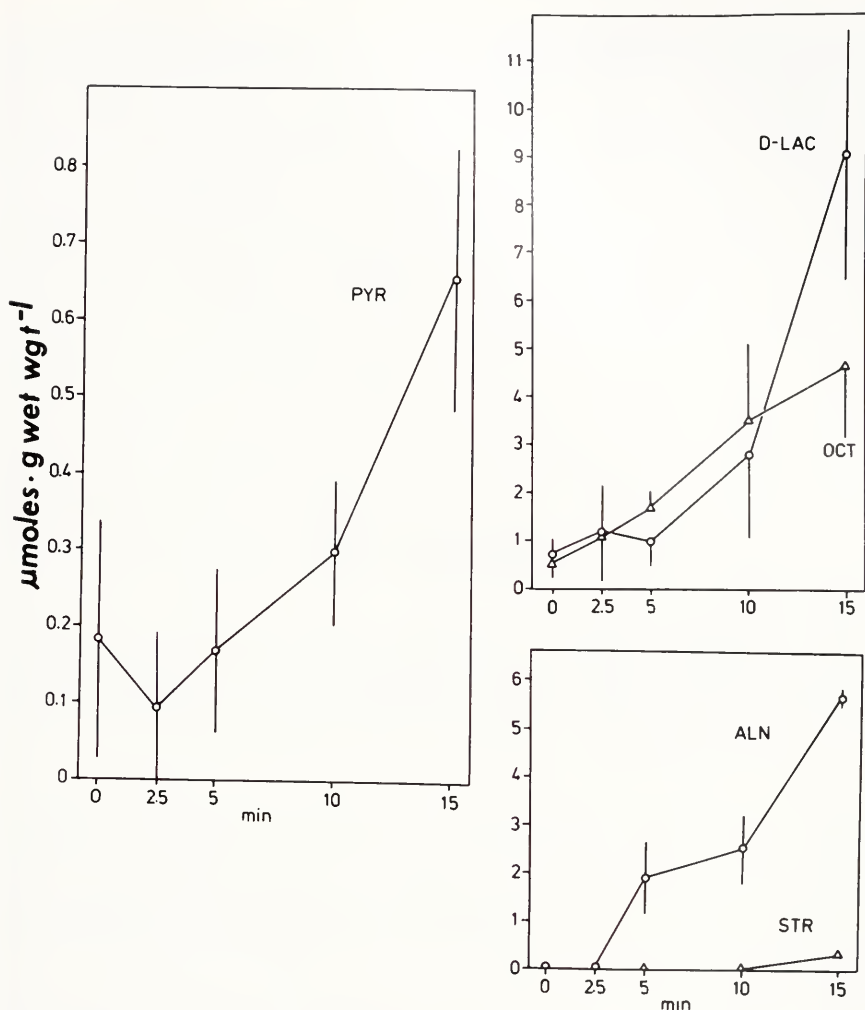


FIGURE 3. Pyruvate (PYR), D-lactate (D-LAC), octopine (OCT), alanopine (ALN), and strombine (STR) levels in electrically stimulated radula protractor muscles from *Busycon contrarium* over time, as determined by HPLC and fluorometry. Units are $\mu\text{moles} \cdot \text{g wet wt}^{-1}$. Points represent means ± 1 SD, $n = 5$.

protractor would undoubtedly enhance all pyruvate reductase activities. In the case of opine dehydrogenases, the concentrations of amino acid co-substrates are also important. In the radula protractor muscle, we found that alanine, glycine, and free arginine levels were in the 2–8 $\mu\text{mole} \cdot \text{g}^{-1}$ range. Finally, pyruvate reductases are influenced by accumulation of their respective products. Opine dehydrogenases seem to be particularly sensitive to product inhibition (Gäde and Grieshaber, 1986).

D-Lactate was the dominant glycolytic end product even though the maximal LDH activity measured in crude tissue extracts was the lowest. Isolated radula muscle of *B. contrarium* displayed the highest levels of D-lactate accumulation yet observed during functional anoxia in molluscs. Meinardus and Gäde (1981) observed a rela-

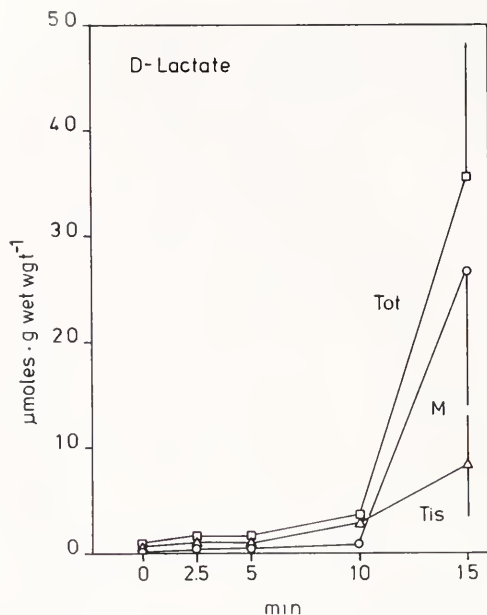


FIGURE 4. Distribution of lactate in the incubation media (M) and tissue (Tis) as well as the total (To) lactate produced during electrically induced isotonic contractions of the radula protractor muscles from the whelk *Busycon contrarium* as determined by fluorometric analysis of neutralized perchloric-acid extracts of each over time. Units are $\mu\text{mole} \cdot \text{g wet wt}^{-1}$. Points represent means ± 1 SD, $n = 5$. Where error bars are absent, the error bar was less than the size of the symbol used to mark points.

tively modest accumulation of D-lactate in electrically stimulated preparations of the foot muscle of the cockle *Cardium edule*. The preferential production of D-lactate *versus* the opines in *B. contrarium* radula protractor muscle may be related to a higher binding capacity for pyruvate. The apparent K_m s for pyruvate of molluscan LDHs are considerably lower than corresponding K_m s of ODHs and somewhat lower than pyruvate K_m s for strombine and alanopine dehydrogenases (Gäde and Grieshaber, 1986).

Isolated radula muscle preparations of *B. contrarium* did not release octopine, alanopine, or strombine into the medium. In addition, the total arginine pool (free arginine, arginine phosphate, and octopine) remained constant during contractile activity. The decline in aspartate levels during anoxia probably reflects transdeamination to alanine because, at the end of 15 min, the alanopine-alanine pool size was roughly equivalent to the decrease in aspartate plus initial alanine levels. This conservation of nitrogen in both the arginine (arginine, arginine phosphate, octopine) and alanine (alanine, alanopine, aspartate difference) pools is consistent with the observed absence of transport of opine end products out of the muscle.

In contrast to that of the opines, release of D-lactate from the muscle was very large. In fact, D-lactate export was greater than D-lactate accumulation during the 10–15-min period of observation. D-lactate release could have at least two major functional advantages. First, removal of the product of this reaction would change the mass action ratio ($[\text{lactate}]/[\text{pyruvate}]$) in favor of more product formation. Second, if lactate were transported out in a symport system with a proton (H^+) or in an antiport system (OH^-), this process would help the muscle cells regulate intracellular pH (pH_i).

The total D-lactate produced during the 15 min of contractile activity approached $50 \mu\text{moles} \cdot \text{g wet wt}^{-1}$. On the basis of established proton stoichiometries of glycolysis (Pörtner *et al.*, 1984)—*i.e.*, one mole of protons (H^+) per mole lactate (or opine) produced—lactate and also opine production clearly impose a significant acid load on the muscle. Buffering capacities of whelk radular and ventricular muscle, as determined by the homogenate titration method (Castellini and Somero, 1981), ranged from 30.7 to 39.5 Slykes $\cdot \text{g wet wt}^{-1}$ (Eberlee and Storey, 1985; Graham and Ellington, 1985). However, the buffering capacity of *B. contrarium* ventricles, as determined by imposing an acid load (Ellington, 1985) and measuring pH_i by phosphorus nuclear magnetic resonance (^{31}P -NMR) spectroscopy, yielded a value approaching 24 Slykes $\cdot \text{g wet wt}^{-1}$ (Ellington, unpub. obs.). Regardless of the exact position of the buffering capacity, the acid load imposed on the muscle clearly could not be offset by purely passive means such as buffering.

Lactate transport has been studied extensively in erythrocytes. Three mechanisms of lactate transport have been identified: (1) non-ionic diffusion, (2) classical anion transport, and (3) monocarboxylate carrier (H^+ symport or OH^- antiport) mediated transport. Deuticke *et al.* (1982) reported at least three parallel pathways of lactate transport in erythrocytes. Lactate transport in erythrocytes has become the paradigm on which mitochondrial (Palmieri *et al.*, 1971), hepatocyte (Fafournoux *et al.*, 1985), and whole-muscle (Mainwood and Worsley-Brown, 1975; Seo, 1984) lactate transport have been modeled. The efflux of lactate from the radula protractor muscle of *B. contrarium* is probably caused by one or several of these mechanisms. Because the pK_a of lactic acid is 3.86, and the intracellular pH of molluscan tissues under a variety of conditions ranges from 7.1 to 6.6 (Ellington, 1983a; Graham and Ellington, 1985), almost all the acid would dissociate into anions; therefore, the rates of non-ionic diffusion should be low. A lactate: H^+ symport (or lactate: OH^- antiport), if present in the radula protractor muscle of *B. contrarium*, could be of critical importance in regulating pH_i , especially during periods of elevated glycolytic rates.

In contrast to lactate, the opines are not released into the medium by the radula protractor muscle of *B. contrarium*. In fact, there is no *direct* evidence for release of opines from any molluscan tissue, although the increase in hemolymph levels during contractile activity in cephalopods (Storey and Storey, 1979; Gäde, 1980) and the decline in the total arginine pool in the mantle muscle during swimming in *S. officinalis* (Storey and Storey, 1979) and *Loligo vulgaris* (Grieshaber and Gäde, 1976) suggest indirectly that octopine is released in these cases. No other study of contractile activity in molluscs has revealed a decline in the total arginine pool or release of opines from the muscle (de Zwaan and Dando, 1984).

Why is lactate readily exported from molluscan muscle cells while opines appear to be retained? Specific transporters for amino acids are present in a wide range of cell types (Preston and Stevens, 1982); thus there is no fundamental impediment with respect to transport of these compounds. Opine formation results in no net increase in the number of osmotically active particles because the amino acid condenses with pyruvate derived from a large polymer (glycogen). In contrast, lactate formation results in an increase in osmotically active particles, as glycogen is broken down into smaller fragments. The lack of disturbance of internal osmolarity has been used as a potential functional explanation for the use of opine dehydrogenases rather than LDH in certain molluscan muscles (Zandee *et al.*, 1980; Fields, 1983). A logical derivative of the argument is that it would be disadvantageous to transport out opine end products. However, accumulated end products represent only a small fraction (<5%) of the pool of osmotically active substances. Thus, removal of end products, or lack thereof, for the sole purpose of cell volume regulation seems unlikely. We favor the

possibility that the lack of export of opines is related to the lack of a mechanism for coupling this movement with regulation of pH_i . In fact, since octopine, alanopine, and strombine have both positive and negative charges at prevailing pH_i conditions, a transport mechanism that could couple H^+ (symport) or OH^- (antiport) movement with opine export would be difficult to envision.

The absence of significant amounts of succinate or malate accumulation makes the fermentation of aspartate during functional anoxia unlikely. Although the carbon skeleton may be unaccounted for, nitrogen is balanced through alanine formation. Most probably, the amino group of aspartate is transaminated ultimately to pyruvate to yield alanine, with the carbon skeleton of aspartate entering the Krebs cycle as malate or oxaloacetate. This hypothesis entails the assumption that there is enough oxygen available to the cells to sustain a significant level of aerobic metabolism, at least during the early phases of contractile activity. The myoglobin content of radula muscle is high (Ball and Meyerhof, 1940; Fange and Mattisson, 1958), and molluscan myoglobins typically have low P_{50} values (Read, 1966). Glycolytic rates, as evidenced by end product accumulation, are low in the early phases of contractile activity, suggesting that energy production is largely aerobic during this period. Presumably, the oxygen used in this period could be derived from an internal oxygen store such as myoglobin. Contractile force decreases in the later portions of the time course while pyruvate concentrations increase, suggesting that there is a transition from aerobic to anaerobic processes. Thus, aspartate may be an aerobic substrate during the early phases of muscle contraction.

To sum up, our studies have shown that during contractile activity in the radula protractor muscle of *B. contrarium*, high glycolytic rates prevail with pyruvate being shunted through all the major pyruvate reductases. Strombine, octopine, alanopine, and D-lactate accumulated in order of increasing levels. D-lactate was the major end product although D-lactate dehydrogenase displayed the lowest *in vitro* activity of all pyruvate reductases. D-Lactate, but none of the opines, was released from the muscle into the incubation medium, with rates exceeding $3 \mu\text{moles} \cdot \text{min}^{-1} \cdot \text{g wet wt}^{-1}$. The removal of lactate from the muscle enhances the mass action ratio in favor of lactate formation, thereby increasing carbon flux through this enzyme. The removal of lactate from muscle cells during contractile activity may also help to regulate pH_i through a H^+ symport or OH^- antiport system. This potential role of lactate transport in the regulation of pH_i is currently the subject of intensive investigation in this laboratory.

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ORIENTATION OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*, ON A SANDY BEACH

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ABSTRACT

Adult horseshoe crabs (*Limulus polyphemus*) spawn on sandy intertidal beaches and then return toward the water. Field experiments demonstrated that beach slope was more significant than vision in this orientation behavior. Both blinded and normally sighted crabs showed rapid seaward orientation on beaches with a seaward slope of approximately 6°. Orientation performance was poor on a flat beach, although sighted crabs slightly out-performed blinded crabs. The observed orientation behavior was correlated with the large numbers of horseshoe crabs which failed to return to the water after spawning on sand bars or similar habitats lacking a slope gradient.

INTRODUCTION

Adult horseshoe crabs (*Limulus polyphemus* L.) migrate every spring into Delaware Bay and other Atlantic coast estuaries to spawn on sandy beaches (Shuster, 1982; Shuster and Botton, 1985). These sublittoral animals find, and amplex with, a mate, migrate to the intertidal zone to deposit and fertilize the eggs, and then return to the sea. This implies the existence of spatial orientation mechanisms at each critical stage of the reproductive cycle. Although little is known about the mechanism of long distance migrations from the continental shelf to estuarine spawning grounds (Botton and Ropes, 1987), Rudloe and Herrnkind (1976) showed that submerged crabs near breeding beaches can orient in response to wave surge. Barlow *et al.* (1982) found that visual cues are important during mating, and Barlow *et al.* (1986) suggest that light may be a significant environmental factor associated with seasonal and diurnal variability in mating activity. Visual stimuli may elicit other behavioral responses including direction and speed of locomotion (Cole, 1923; Northrup and Loeb, 1923; Ireland and Barlow, 1978) and telson and gill movements (Powers and Barlow, 1985).

The orientation cues which enable horseshoe crabs to return to the water after mating have not been previously considered. This behavior has important ecological consequences because animals "stranded" on an exposed intertidal beach are subjected to physiologically stressful conditions, including high temperatures, desiccation, and osmotic imbalance (Herrnkind, 1983). Among shore zone arthropods, both visual cues (e.g., sun, moon, polarized light, landmark orientation) and nonvisual cues (such as beach slope) are known (Herrnkind, 1972, 1983; Schöne, 1984). In 1985 during a preliminary beach survey of Delaware Bay in the area of Fortescue, New Jersey, we observed large numbers of live horseshoe crabs burrowed on a relatively flat section of exposed intertidal beach at low tide. We hypothesized that this aberrant

behavior resulted from the inability of the animals to orient seaward in the absence of beach slope; in this report, we present an experimental test of that hypothesis. Orientational behavior of sighted and blinded individuals on sloped and flat beaches were quantified to evaluate the importance of beach slope and light for adult horseshoe crabs.

MATERIALS AND METHODS

All experiments were conducted between 26 May and 7 June 1986 at the New Jersey Oyster Research Laboratory (NJORL), located on the Delaware Bayshore in Cape May County, New Jersey. The study area was located just north of the laboratory. One site had a slope and sediment composition typical of this relatively uniform and undisturbed beach (Shuster and Botton, 1985). During spring, these beaches have a slope of some 6.4 degrees in approximately a westerly direction. The second site was a flat sand bar formed by the outlet of a salt marsh creek. It was located approximately 100 m north of the sloped beach site, also with the bay toward the west. Horseshoe crabs spawned in large numbers in this and adjacent areas during full moon high tides. Both sites within the study area provided similar visual fields: an open horizon in the seaward direction and a line of vegetation (mainly *Phragmites*) above the high water line in the landward (easterly) direction.

Orientation was studied within "arenas" modeled after Mrosovsky and Shettleworth (1968). On each beach, a 4-m radius circle was inscribed and a 30-cm trench dug along its perimeter. Stranded horseshoe crabs and any large pieces of debris were removed from the arena before use. The arena was divided into 16 equal sectors of 22.5° each. Sector 1, beginning at compass point north, was arbitrarily designated as 0°, sector 2 began 22.5° in a clockwise direction, *et seq.*

Adult male horseshoe crabs were collected from the bay immediately before the study of their behavior. Crabs with missing appendages, or with damaged, missing, or heavily encrusted eyes, were discarded. Those crabs kept out of water for more than 3 minutes were thoroughly wetted down before the trial. The animals used in the blinding experiments were prepared by drying the carapace around the lateral and median eyes using a paper towel, and then placing patches of opaque adhesive duct tape over the eyes.

Each of the four combinations of beach slope and vision, *i.e.*, sloped beach/sighted, sloped beach/blinded, flat beach/sighted and flat beach/blinded, were run on at least two separate days, with a total sample size of not less than 41 individuals per combination (range, 8–23 crabs per run). Crabs were tested individually to avoid behavioral interactions because they often pause or change direction when other crabs are encountered along the beach (pers. obs.). To begin a trial, the top few cm of sand in the arena was smoothed using a wooden board to remove the track of the previous animal; this procedure also disrupted any possible gradients in surface sediment moisture. The crab was placed in the center of the arena facing away from the water and on a line perpendicular to the shoreline. If the animal burrowed in place, it was lifted out of the sand and re-started; if it burrowed twice in succession, it was rejected.

A trial was completed when the animal's prosoma crossed the perimeter of the arena. Animals failing to complete a trial, or which burrowed, or which remained motionless on the surface of the sediment for 5 consecutive minutes were recorded but not used in the statistical analysis of this behavioral data set. Investigators recorded the elapsed time of the test, the section of the arena from which the crab exited, and the number of pauses longer than 30 s. The linear distance of the path was measured by placing a metered string along the animal's track; a sketch of the path for each trial was made.

TABLE I

Comparison of the number of "disoriented" (live, rightside-up) Limulus polyphemus on flat and sloped beaches in Delaware Bay on each of three days during Spring 1986

Date	Mean no. disoriented crabs per 15-m transect		n	t
	Flat	Sloped		
23 May	55.2	12.2	5	4.552**
24 May	93.3	9.8	4	3.342*
25 May	101.5	4.5	4	3.623*

n = number of replicate 15-m transects counted on each type of beach, t = t-statistic comparing mean number of crabs on each type of beach. Means on flat beach significantly greater than mean number on sloped beach at $P < .05$ (*) or $P < .005$ (**).

The performance of a group of crabs was assessed using a number of variables including percent crossing the perimeter, time required to leave the arena, linear distance travelled, and the number of pauses and circles made by the animals. We calculated each animal's "meandering score" as the linear distance of the path divided by the radial distance (4.0 m) (Mrosovsky and Kingsmill, 1985). We computed mean vectors, mean angles of orientation, and 95% confidence intervals for each group of animals (Zar, 1984, p. 428). All animals were assumed to cross at the midpoint angle in the appropriate sector. The Rayleigh test (Zar, 1984, p. 443) examined the null hypothesis that there was no mean population direction. Differences between mean angles were analyzed using the Watson-Williams procedure (Zar, 1984, p. 446).

We surveyed the "disoriented" crabs in transects on the flat and sloped beaches on 23–25 May. Disoriented crabs were defined as those live animals remaining rightside-up on the breeding beach at low tide, as distinguished from the normal behavior, which is to follow the receding tide and spend the low tide period on the intertidal sand flat. Live upside-down crabs, "stranded" by wave action, were not counted.

RESULTS

Surveys of disoriented crabs

The number of disoriented (live, rightside-up) stranded crabs was significantly higher on the flat beach than the sloped beach on each of the three days (Table I). Since similar, or perhaps slightly lower numbers of mating horseshoe crabs actually approached the flat beach compared with nearby sloped beaches (based on Botton, 1982 and personal observations during the 1986 field season), the accumulation of disoriented crabs on the flat beach is not likely to be a numerical artifact. Similar dense concentrations of disoriented crabs were observed near creek mouths along Delaware Bay north of our study area during a survey in 1985. In contrast, only about 20–25% of live crabs stranded on sloped beaches during May, 1986 were rightside-up (disoriented) animals; the remainder were stranded upside-down as a result of wave action and/or telson abnormalities (R. E. Loveland and M. L. Botton, unpub. data). Approximately 24–34% of the disoriented individuals on the flat beach were stranded as mated pairs (males amplexed to females). By comparison, only 46 disoriented mated pairs (of a total of 4247 stranded crabs) were found on two sloped 90-m study beaches between 15 May and 21 June 1986 (R. E. Loveland and M. L. Botton, unpub. data).

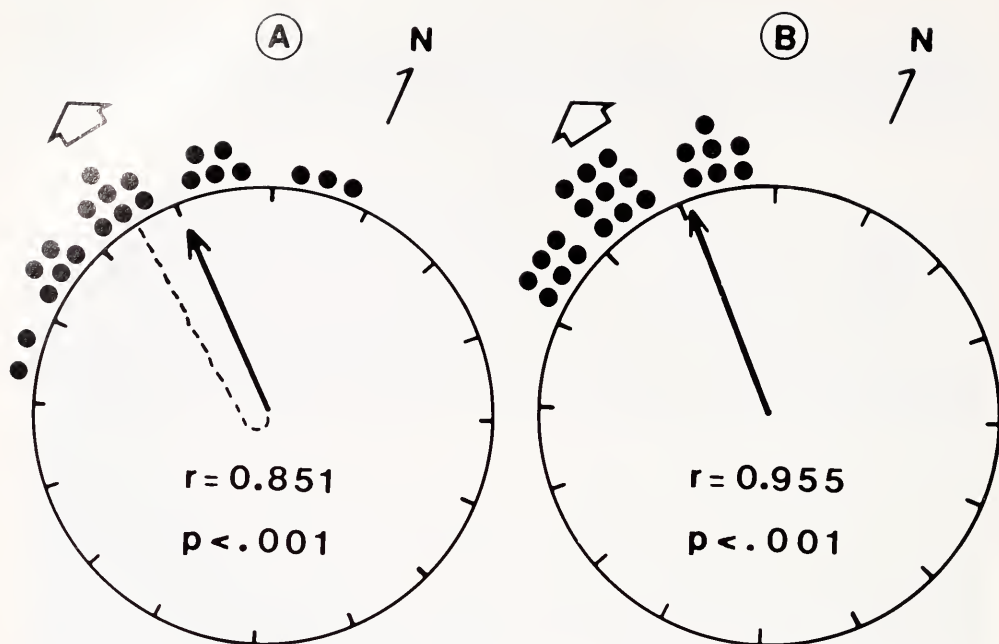


FIGURE 1. Orientation behavior of horseshoe crabs with normal vision on a sloped beach. Run A held 26 May, 1610–1850, bright sunlight, wind W less than 5 mph. Run B held 2 June, 1057–1323, cloudy with occasional drizzle, wind NNE 15 mph. Length and direction of mean vector r shown by solid arrow; Rayleigh's Test indicated significant mean population direction in both runs. Seaward direction indicated by open arrow. Typical "fish hook" path followed by a crab is shown in A.

Orientation on sloped beach. All sighted crabs showed strong seaward orientation (Fig. 1). The path followed by nearly all crabs in these trials was the "fish hook" pattern illustrated in Figure 1a. Typically, a crab first walked in the direction it was placed (in this case, uphill) before turning to its left or right. More crabs turned left ($n = 26$) than right ($n = 16$) but the difference was non-significant ($\chi^2 = 2.38$, $.25 < P < .10$). There was no significant difference in mean angle between a group of crabs run in late afternoon ($n = 23$) and a second group ($n = 23$) run in midday (Watson-Williams test, $F = 0.229$, n.s.). In both trials, 22 animals (96%) completed the test and there was no significant difference in the time it took to complete the test (Mann-Whitney U-test, $z = 0.493$, n.s.). The meandering score was significantly lower in the late day group ($U = 365.0$, $P < .005$). Pausing and circling behaviors were noted only five times each (Table II).

Blinded crabs also showed strong seaward orientation on the sloped beach (Fig. 2). Mean angles of crabs tested in early morning and mid-afternoon were virtually identical. In both the morning and afternoon experiments, 20 animals (87%) completed each trial. Animals in the morning trial took significantly longer (Mann-Whitney U-test, $U = 352.5$, $P < .001$) and had a higher meandering score ($U = 316$, $P < .002$) than the afternoon group. This difference was probably related to a steepening of the beach slope before the afternoon trial, which was caused by strong wave action several tidal cycles earlier. Blinded animals followed the typical fish hook path described above; the direction of turning was random (20 to the left, 21 to the right). No blinded animal circled in either trial and pausing was infrequent (Table II).

Orientation on flat beach. Horseshoe crabs with normal vision had difficulty ori-

TABLE II

Measures of orientation performance (means and standard errors = SE) of horseshoe crabs on sloped and flat beaches

Variable	Sloped beach				Flat beach					
	Normal vision		Blinded		Normal vision			Blinded		
	A	B	A	B	A	B	C	A	B	C
Time to completion	88.9	103.3	134.7	58.1	271.9	128.8	192.1	183.2	395.7	374.9
SE	11.8	16.4	19.1	5.6	53.5	37.6	22.0	61.3	137.2	64.8
Meandering score	1.21	1.43	1.48	1.37	2.36	2.15	2.01	1.72	2.04	3.45
SE	0.03	1.13	0.04	0.07	0.49	0.32	0.15	0.27	0.28	0.37
No. of pauses	0.04	0.26	0.22	0.17	0.50	0.50	0.13	0.75	1.87	1.06
SE	0.04	0.12	0.09	0.10	0.20	0.31	0.07	0.41	0.62	0.36
No. of circles	0.09	0.13	0.00	0.00	1.00	0.60	1.17	0.75	0.67	2.33
SE	0.06	0.07	0.00	0.00	0.35	0.31	0.31	0.31	0.21	0.52
Number completing	22	22	20	20	11	8	21	6	6	13
Number burrowing	0	0	1	1	4	1	0	1	4	2
Number stopped without burrowing	1	1	2	2	1	1	2	1	5	3
Total number in run	23	23	23	23	16	10	23	8	15	18

Time to completion (in seconds) and meandering scores based on only those animals crossing the perimeter of the 4-m radius testing arena. Letters at top of column designate individual runs.

enting on the flat beach. Three separate runs were conducted. In afternoon runs under both overcast and sunlight conditions, values of Rayleigh's z indicated that crabs were not significantly oriented in any direction (Fig. 3a, b). In the third run, on a sunlit morning, there was significant orientation in a seaward direction (Fig. 3c). However, four animals which exited through a seaward-facing sector were actually travelling parallel to the shoreline when they crossed the perimeter.

Sighted crabs on the flat beach had a 10-fold increase in pauses and number of circles, and higher meandering scores, compared with animals on the sloped beach (Table II). Nine of the 40 animals (22.5%) tested in the three runs either burrowed or stopped moving for 5 minutes, thus failing to complete the test.

Horseshoe crabs behaved differently on the flat beach than on the sloped beach, although variability was high among those tested. Very few paths formed the fish hook pattern described earlier. Initially, crabs moved rapidly in the direction they were placed, but typically they turned and slowed down within the first meter. Many crabs reared up on their pedipalps and moved slightly from side to side. This behavior was often followed by circling, which in most cases began within 1 m of the release point, although some animals made wider loops, "figure 8's," or both (Fig. 3a).

Blinded crabs were even more disoriented on the flat beach than were the sighted individuals. The percentage of crabs completing the test was the lowest of the four experimental combinations (Table II). Seven of the 41 crabs (17%) burrowed before crossing the perimeter and another 9 (22%) stopped moving without burrowing. Of those crossing the perimeter, the direction was random (Fig. 4). There was no significant difference in the time to complete a trial between the three groups; however, crabs in the third run had a significantly higher meandering score than those in the first two.

In comparing sighted and blinded crabs on the flat beach, the blinded animals took longer ($\bar{x} = 333.8$ s) than sighted animals ($\bar{x} = 201.4$ s) to complete the test ($U = 663$, $P < .05$). The meandering scores were not significantly different between the

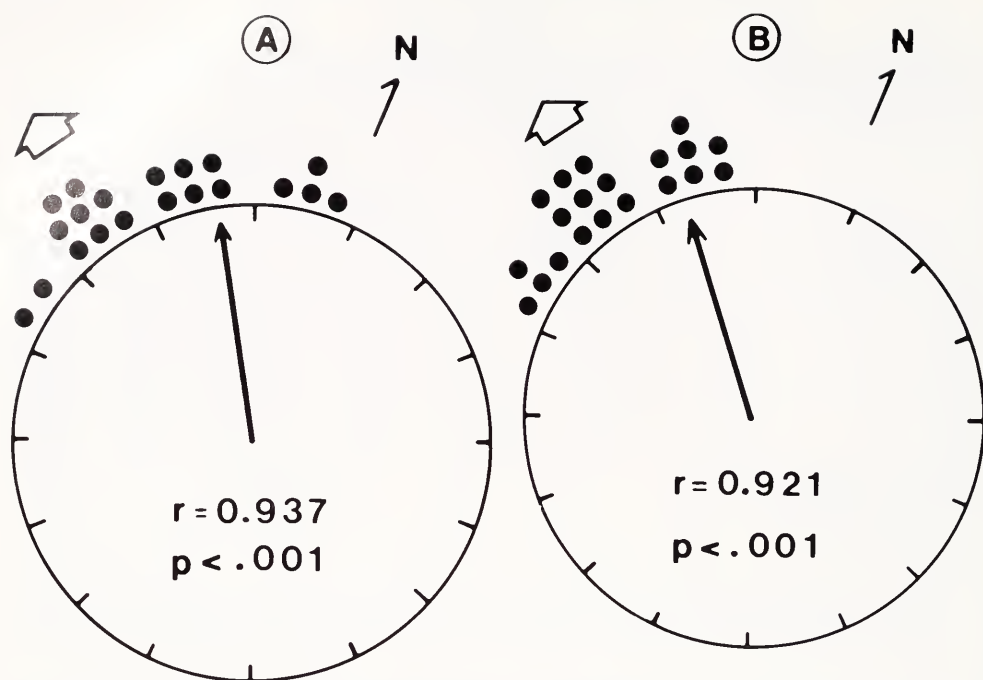


FIGURE 2. Orientation behavior of blinded horseshoe crabs on a sloped beach. Run A held 28 May, 0710–1038, heavy cloud cover with mist, wind WSW 5 mph. Run B held 4 June, 1250–1600, bright sun, wind S 10 mph; slightly steeper slope than Run A because of strong wave action during previous 48 hours. Length and direction of mean vector r shown by solid arrow; Rayleigh's Test indicated significant mean population direction in both runs.

sighted crabs and the first two groups of blinded crabs, but both were significantly lower than the third group of blinded crabs (Kruskal-Wallis test, $H = 11.54$, $P < .005$).

DISCUSSION

As noted by Herrnkind (1983), orientation by shore zone arthropods could potentially involve visual and/or nonvisual guideposts. Among arthropods, visual cues are important in various amphipods, isopods, decapods, insects, and wolf spiders (Herrnkind, 1972). In contrast, beach slope has been demonstrated to be involved in orientation far less frequently (Hamner *et al.*, 1968; Craig, 1973). At our Delaware Bay study area, there appears to be a strong visual contrast between the dark horizon in a landward direction and the open horizon in the bayward direction; therefore, we considered the possibility that horseshoe crabs on sandy beaches might be employing vision.

There is considerable precedent in the literature concerning the behavioral responses of *Limulus* to light. Cole (1923) found that asymmetrically blinded animals 20–60 mm in diameter (which he erroneously considered to be adults) circled most frequently in the direction of the remaining lateral eye. Northrup and Loeb (1923) found that young crabs (*ca.* 15 cm length) were negatively phototropic. *Limulus* can also detect polarized light (Waterman, 1950), but at present there is no experimental evidence implicating this in any known behavioral response. More recently, Barlow and collaborators (*e.g.*, Barlow *et al.*, 1980) have shown the presence of a circadian

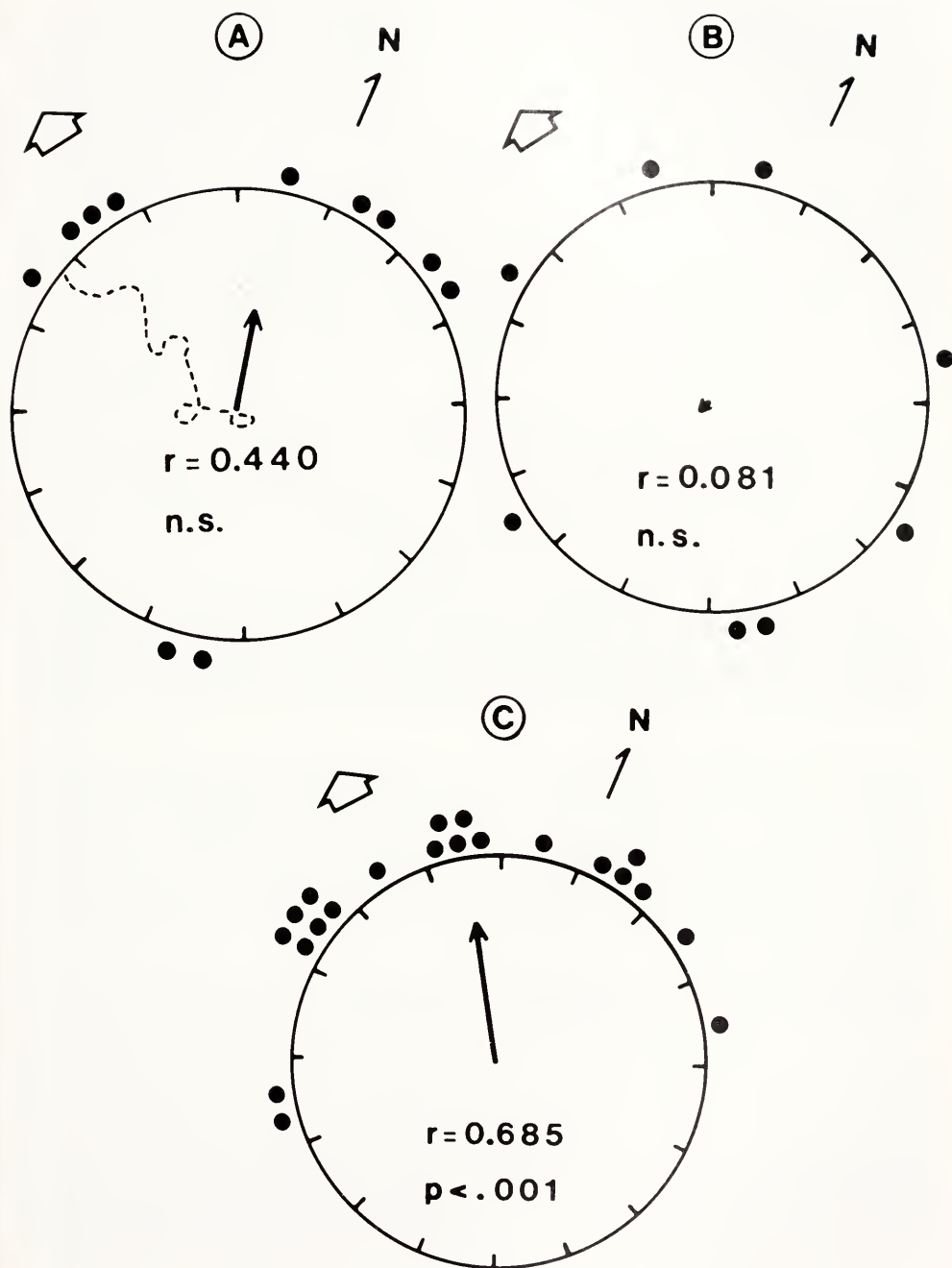


FIGURE 3. Orientation behavior of horseshoe crabs with normal vision on a flat beach. Run A held 27 May, 1555–1930, heavy cloud cover, wind calm. Run B held 28 May, 1622–1747, bright sun, wind WSW 5 mph. Run C held 5 June, 0825–1200, bright sun, wind S 5 mph. Length and direction of mean vector r shown by solid arrow; Rayleigh's Test rejected the null hypothesis of no mean population direction in Run C only. Seaward direction indicated by open arrow. Typical path followed by a crab is shown in A.

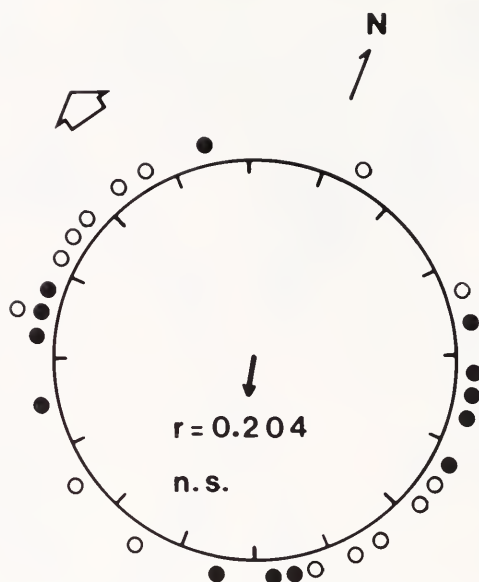


FIGURE 4. Orientation behavior of blinded horseshoe crabs on a flat beach. Runs A (30 May, 1314–1450, wind W 5 mph) and B (31 May, 0748–1205, wind W 5 mph) were combined (open circles) because of the small number of animals completing each trial. Run C (7 June, 0730–1245, mostly sunny, wind S 5 mph) shown with filled circles; length and direction of mean vector r (solid arrow) based on Run C data. Population showed random orientation. Seaward direction indicated by open arrow.

rhythm in ommatidial morphology and visual sensitivity, modulated by a clock in the animal's brain. Visual sensitivity is higher at night, enabling males to recognize female "models" even during the evening (Barlow *et al.*, 1982). Blinded crabs released in the subtidal environment were more disoriented than sighted crabs (Ireland and Barlow, 1978). However, both blinded and sighted submerged crabs were capable of orienting in the vicinity of the breeding beach when wave surge was present (Rudloe and Herrnkind, 1976).

Experiments with blinded and sighted crabs on both sloped and flat beaches indicate that beach slope, not visual stimuli, is the primary cue used by horseshoe crabs to return to the water. Both blinded and sighted animals showed rapid seaward orientation on a sloped beach. In contrast, orientational performance was severely impaired on a spawning beach lacking slope. Sighted and blinded crabs showed pausing and circling behaviors on the flat beach far more frequently than on the sloped beach. Circling, accompanied frequently by the animal's rearing up on their pedipalps, may have been an attempt to obtain directional information from gravitational cues. Since these directional cues were lacking in the flat arena, it is not surprising that meandering scores on the flat beach were higher, and many animals either burrowed, stopped moving entirely, or left the arena on a heading that took them away from the water.

The physiological basis for the observed response to beach slope is not clear. Despite the extensive use of *Limulus* in neurophysiological research, no statocyst or other balance organ has been described. However, proprioceptors have been identified from the gnathobases and joints of the walking legs (Barber, 1956, 1960; Pringle, 1956; Barber and Hayes, 1964). We hypothesize that stimulation of these receptors in a crab walking "uphill" may elicit a turning response which then directs crabs down the slope, toward the water. A crab can presumably detect when it has started

"downhill," since once on this heading, turns are infrequent. The information registered by these proprioceptors would be constant on a flat beach, which may underlie their lack of orientation. It seems unlikely that mechanoreceptors on the dorsal carapace (Kaplan *et al.*, 1976) or lateral spines (Eagles, 1973) are involved in beach orientation, although they may provide positional information under different circumstances.

The comparison between the responses of blinded and sighted animals on the flat beach suggests a possible secondary role for vision in sandy beach orientation. One of three groups of sighted crabs showed significant seaward orientation (Fig. 3), but why this group did, and the other two did not, is not apparent. Overall, sighted crabs on the flat beach exhibited somewhat better orientation (as measured by percent finishing, number of pauses, and meandering scores) than the blinded crabs on the same arena, although their performance was much poorer than the blinded crabs on the sloped beach.

A comparable and ecologically similar orientation problem confronts female sea turtles after egg laying. In these animals, in contrast to *Limulus*, the primary cues are visual. Females, as well as hatchlings, assess the brightness differential between the open, seaward horizon and the darker, vegetation-lined landward horizon (Ehrenfeld and Carr, 1967; Mrosovsky and Carr, 1967; Mrosovsky and Shettleworth, 1968; Mrosovsky, 1970). In comparison, there is some evidence for positive geotropism among hatchlings (Parker, 1922; Van Rhijn, 1979), although Mrosovsky and Kingsmill (1985) point out that on many turtle nesting areas, slope is very irregular and not as good a predictor of seaward direction as is the open horizon. This configuration is markedly different at the Cape May shore of Delaware Bay, where undisturbed beaches consistently have a seaward slope. A positive geotaxis is therefore a reliable orientation behavior for adult horseshoe crabs, whereas for sea turtles, it is not. However, it might be adaptive for a species to have a secondary orientation mechanism should the primary system fail. For sea turtles, geotaxis may function, albeit weakly, when vision is impaired (Van Rhijn, 1979); a similar hierarchy of cues exists in the amphipod, *Orchestoidea corniculata* (Herrnkind, 1983). For horseshoe crabs, vision may be of some limited value when the crabs become stranded on a flat beach, but many animals which spawned on this type of beach are unable to find the water only a few meters away (Table I). Under these circumstances, it seems adaptive for horseshoe crabs to burrow because it conserves energy and keeps their book-gills in contact with cooler, moist sand. If high tide during the next day is of equal or greater amplitude than the one on which they were stranded, the crabs will have a good chance of survival, but if tidal heights are declining, there may be localized, large-scale mortality.

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THE FEEDING BEHAVIOR OF *PARANOPHRYS CARNIVORA* (CILIATA, PHILASTERIDAE)

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ABSTRACT

The marine ciliate *Paranophrys carnivora*, isolated from the Mediterranean coast of Israel, was found to feed on a varied diet of bacteria, algae, and living and non-living tissues. *Chlorococcum* sp. and *Dunaliella parva*, the algal species on which *P. carnivora* grew best, did not elicit a chemosensory response; tissues and bacteria did. In experiments on stationary phase ciliates, betaine, choline, L-histidine, and trimethylamine oxide elicited a positive chemosensory response at concentrations as low as 10^{-6} M to 10^{-3} M.

INTRODUCTION

Most ciliates feed on particulate matter consisting mainly of microorganisms (bacteria and algae) of a size appropriate to their buccal apparatus. The particles, whether suspended or settled, are collected via specialized cilia near the oral opening (Corliss, 1979).

In *Tetrahymena*, one of the ciliates most studied, particulate matter seems obligatory for feeding. An autoclaved 2% proteose peptone medium which supports a flourishing culture, loses this capability when the particles are removed by millipore filtration. Addition of inert particles lacking any nutritive value to the filtered medium restores its growth potential (Rasmussen and Kludt, 1970; Rasmussen and Modeweg-Hansen, 1973). Extensive experiments performed by Fenchel (1980a, b, c) with inert "latex" particles indicate that various ciliates select their food primarily by particle size.

When offered different combinations of algal species of the same size range, the ciliate *Favella ehrenbergii* showed a preference for one species, indicating that food selection is also based on factors other than size (Stoecker *et al.*, 1981). Selective feeding has been attributed to chemical stimuli in various other ciliates *e.g.*, *Nassula* (Poilvert, 1959), *Stentor coeruleus* (Tartar, 1961; Rapport *et al.*, 1972), and *Podophrya calkinsi* (Hull, 1961). In addition, particle movement affects the feeding behavior of ciliates (Karpenko *et al.*, 1977).

Most studies on the feeding behavior of ciliates focused on those feeding on microorganisms *e.g.*, *Paramecium*, *Tetrahymena* (Levandowsky and Hauser, 1978; Van Houten *et al.*, 1981, 1982; Antipa *et al.*, 1983; Levandowsky *et al.*, 1984; Leick and Hellung-Larsen, 1985; Hellung-Larsen *et al.*, 1986). Studies on ciliates that feed on tissues are scarce (Levandowsky and Hauser, 1978; Van Houten *et al.*, 1981). The marine ciliate *Paranophrys carnivora*, which was recently described, feeds on tissues of living or dead organisms (Czapik and Wilbert, 1986). The feeding behavior of this organism is described in this paper.

MATERIALS AND METHODS

Cultivation and morphology of Paranophrys carnivora

P. carnivora was isolated from samples collected from the Mediterranean Sea at the coast of Dor, Israel. The samples were rich in various protozoans: Ciliata e.g., *Euplotes*, and Flagellata, mostly autotrophic ones, including *Dunaliella*. Initial observations showed that *P. carnivora* fed on algae. They were also observed to gather in the vicinity of freshly injured invertebrates (crustaceans) and feed on their tissues.

Several *P. carnivora* clones were prepared and grown on *Dunaliella parva* as well as on a strain of the bacterium *Enterobacter aerogenes* which can grow at a salinity of 35%. No attempt was made to eliminate the original bacterial flora. The most successful clone was further cultured on *E. aerogenes* for growth curve and feeding behavior studies. These bacteria were grown on brain heart Agar (Difco) slants at 28°C, and then harvested in sterile water to give a suspension having an absorbance of 1.1–1.3 at 430 nm, as measured with a Bausch and Lomb "Spektronic 20" spectrophotometer. A 2-ml inoculum of a 3–4 day-old culture of *Paranophrys* and 0.4 ml of the bacterial suspension were added to a test tube (3 cm diameter, 20 cm length) containing 30 ml of sterile (autoclaved) 35‰ artificial seawater (Instant Ocean salts from Aquarium Systems, Mentor, Ohio, in filtered water, hereafter referred to as ASW). The culture was then incubated at 28°C in a temperature-controlled chamber. For growth curve studies, 0.5- or 1-ml samples were removed at various intervals from each of three cultures which had been inoculated simultaneously from the same source, and preserved in a 10% Bouin solution. All of the ciliates in each sample were counted in a glass chamber at 60× with a hand tally. Size determinations were made at 600× using an ocular micrometer.

The morphological description and identification was based on the same clone which was kept in 15 ml of ASW in covered glass vials at 20°C and fed every 14 days on 3 pin-head sized bits of either oligochaete or crustacean meat. Biometric measurements were made using a light microscope on ciliates stained by the protargol (Wilbert, 1975) and silver nitrate (Chatton and Lwoff, 1930) methods. For scanning electron microscopy, cells were fixed instantaneously by rapid addition of a large volume of 2.7% OsO₄ in ASW. After 10–15 minutes in fixative at room temperature, the cells were washed with 2% glutaraldehyde in ASW. After 10 minutes they were dehydrated in a graded ethanol series, dried by the critical point method, coated with gold-palladium, and viewed in a Joel 840 scanning electron microscope.

Feeding and growth experiments with marine algae

The species of marine algae whose names are given in Table II were cultivated in test tubes in a medium of ASW enriched with Walne solution (Walne, 1966) in a temperature-controlled room at 18°C and under continuous illumination. Young, flourishing week-old cultures were inoculated with *Paranophrys carnivora* and further incubated under continuous illumination at 25°C. The cultures were examined with a dissecting microscope at 40×—both initially and at various intervals during a week—to appraise the ciliate population growth. Samples were also examined under higher power of the light microscope, while alive and after they were killed with a 1% formalin solution. At 60×, the dimensions of the algae (length/width or diameter) were determined using an ocular micrometer. Ciliates were also examined for vacuoles containing algae.

Behavior experiments

Capillary tube assay. The amino acids tested were purchased from Sigma (L-leucine, L-isoleucine, L-proline, hydroxy-L-proline, L-arginine, D- and L-histidine,

L-cysteine); Nutritional Biochemical Corporation (DL-phenylalanine, DL-alanine, DL-serine, L-methionine, L-threonine, DL-asparagine); British Drug House, Ltd. (glycine, L-aspartic acid); Merck & Co., Inc. (L-tyrosine); Light & Co., Ltd. (L-cystine); Fluka (DL-valine); and CHR (L-tryptophan). Betaine hydrochloride, choline chloride, and trimethylamine oxide (TMAO) were also purchased from Sigma, and proteose peptone and brain heart infusion from Difco Laboratories.

The substances to be tested were dissolved in distilled water and the pH of the solution was adjusted, if necessary, to the pH of ASW (pH 8). Glass capillary tubes (Modulohm of Helver, Denmark) 5–8 mm long and 0.7–1.0 mm in external diameter, were filled with the test solution and dried. In a previous study with another species of the same family, *Porpostoma notatum*, many ciliates would enter a control capillary containing fresh medium even when the medium they were swimming in was only half-an-hour old (Kahn *et al.*, 1981). Therefore we modified the capillary assay by drying the test solutions and then, during the experiment, allowing the test substances to become dissolved in the same medium the ciliates were swimming in, rather than use fresh medium as a solute. Control capillary tubes were not filled with any chemicals. Tissue culture dishes, 35 × 10 mm (Falcon), were each filled with 2 ml of ciliates from the stationary phase of culture, which had been diluted with fresh ASW to a density ranging from 100 to 300 ciliates per ml. For each concentration and substance to be tested, a test and a control tube were placed in different halves of each experimental dish. When both test and control dry capillaries were immersed in the ciliate suspension at the start of each experiment, they became filled with the medium in which the ciliates were suspended. The test substances were dissolved within the experimental period (the substances found later to elicit a positive chemosensory response were further tested separately in a series of identical capillaries. These substances dissolved completely within a five-minute period). In each experimental run, a test and control capillary pair were tested in each of three dishes *i.e.*, in triplicate. Betaine, found earlier to elicit a chemosensory response from *Paranophrys carnivora* (mistakenly identified as *P. magna*; Kahan *et al.*, 1985), was used at a concentration of 10^{-1} M (together with a control capillary) as a standard in each experimental run to verify the responsiveness of the ciliates. These betaine and control pairs were also run in triplicate. Using a dissecting microscope, the number of ciliates in each tube (up to about 100) was recorded with a hand tally at intervals during a 30-minute period. Levandowsky *et al.* (1984) preferred using flat capillaries to eliminate difficulties encountered in viewing *Tetrahymena* ciliates through cylindrical capillaries. We did not experience difficulty in counting moving *Paranophrys carnivora* in the cylindrical tubes. In the initial screening, most of the amino acids were tested at a concentration of 10^{-1} M, with the exception of L-glutamic acid and L-tryptophan, at 5×10^{-2} M, and L-tyrosine, at 2×10^{-3} M. L-histidine was the only amino acid which elicited a positive response at least as strong as that of betaine. This amino acid, as well as betaine, choline, and trimethylamine oxide (TMAO), was further tested in at least four experimental runs at concentrations from 10^{-6} or 10^{-4} to 10^{-1} M.

The chemosensory response was computed at each time interval as the ratio of the number of ciliates in the tube containing the test substance to the total number of ciliates in both the test and control tubes. Since statistical analysis (via *t*-tests) of the differences in response at different times during each half-hour experimental run showed no consistent effect of observation times, the index of chemosensory response for a given experimental run was defined as the maximum of the chemosensory responses at the time intervals measured. To adjust for variation in chemotactic responsiveness over the different days of the experimental runs, a relative index of chemotactic activity for a given substance at a given concentration, was defined as the ratio of

its chemotactic activity to the index of the standard (betaine, at a concentration of 10^{-1} M) for the same experimental run.

Both the index and relative index of chemosensory response of *P. carnivora*, for the various substances at different concentrations, were analyzed by two-way analysis of variance (Scheffé, 1959). Effects on the index due to substances or concentrations were analyzed using the S-method of multiple comparisons (Scheffé, 1959).

Dialysis experiments. Dialysis bags, 20 cm in length and 1.6 cm in diameter (Visking Tubing, The Scientific Instrument Center, Ltd.), were filled with 10 ml of either test solution (5% proteose peptone in ASW) or control (ASW alone). They were immersed in separate finger bowls each containing 150 ml of ciliate suspension at a density of 40 per ml. This was prepared by diluting a stationary phase culture with fresh ASW. To evaluate the behavioral effect, the tubing was first examined along its entire length—using the low magnification of the dissecting microscope—for the greatest congregation of ciliates. This section was further examined under 40× and the number of ciliates on both the test and control bags was compared at consecutive time intervals for up to 2 hours.

RESULTS

The growth curve of Paranophrys carnivora and associated morphological changes

The growth curve of *P. carnivora* fed on *Enterobacter aerogenes* at 28°C is shown in Figure 1. Figure 1 shows that the logarithmic growth phase continues for up to about 30 hours with a generation time of 7–8 hours. The stationary phase which follows is short, and after 48 hours there is a moderate decline in the number of ciliates. This phase continues until the experiments are terminated at the end of the fifth day. During the growth experiments the shape of the cell changed from ovoid (the “trophic” form, having a length to width ratio of 1.8 in the logarithmic phase) to more elongated (the “swimming” form, with a ratio of 2.2 or more in the stationary and decline phases). More pronounced differences between the two forms were obtained with cultures fed on either oligochaete or crustacean meat and, rarely, from cultures fed on algae. Scanning electron micrographs of the two forms from cultures fed on *Dunaliella parva* are shown in Figures 2 and 3. The biometric data presented in Table I are of silver stained specimens from cultures fed on oligochaete or crustacean meat, as are the light micrographs given in Figures 4 and 5. In Figure 5, note the marked appearance of the stained kinetosomes and the protrichocysts, another characteristic of the swimming form.

Feeding and growth experiments with marine algae

Table II shows that *Paranophrys carnivora* ingested most of the algae offered. However, different results were obtained with the various algal species ingested. The best growth was obtained with *Chlorococcum* sp. and *Dunaliella parva*; no growth occurred with *Chlorella saccharophila* and *Dunaliella primolecta*. As might be expected, those algal species that were not ingested did not support good ciliate cultures.

Chemosensory response

In the laboratory, *P. carnivora* fed on either *Enterobacter aerogenes*, various algae, or wounded *Artemia*, dead or alive, when each of these diets was offered individually. Differences in chemosensory responses were obtained when capillary tubes containing one of the diets was offered with a capillary containing no food (control), and the number of ciliates in each of the two tubes compared after 10 minutes. As shown in

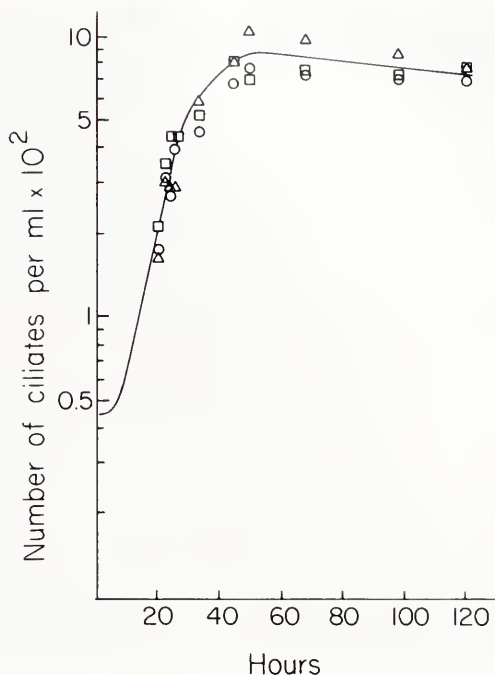
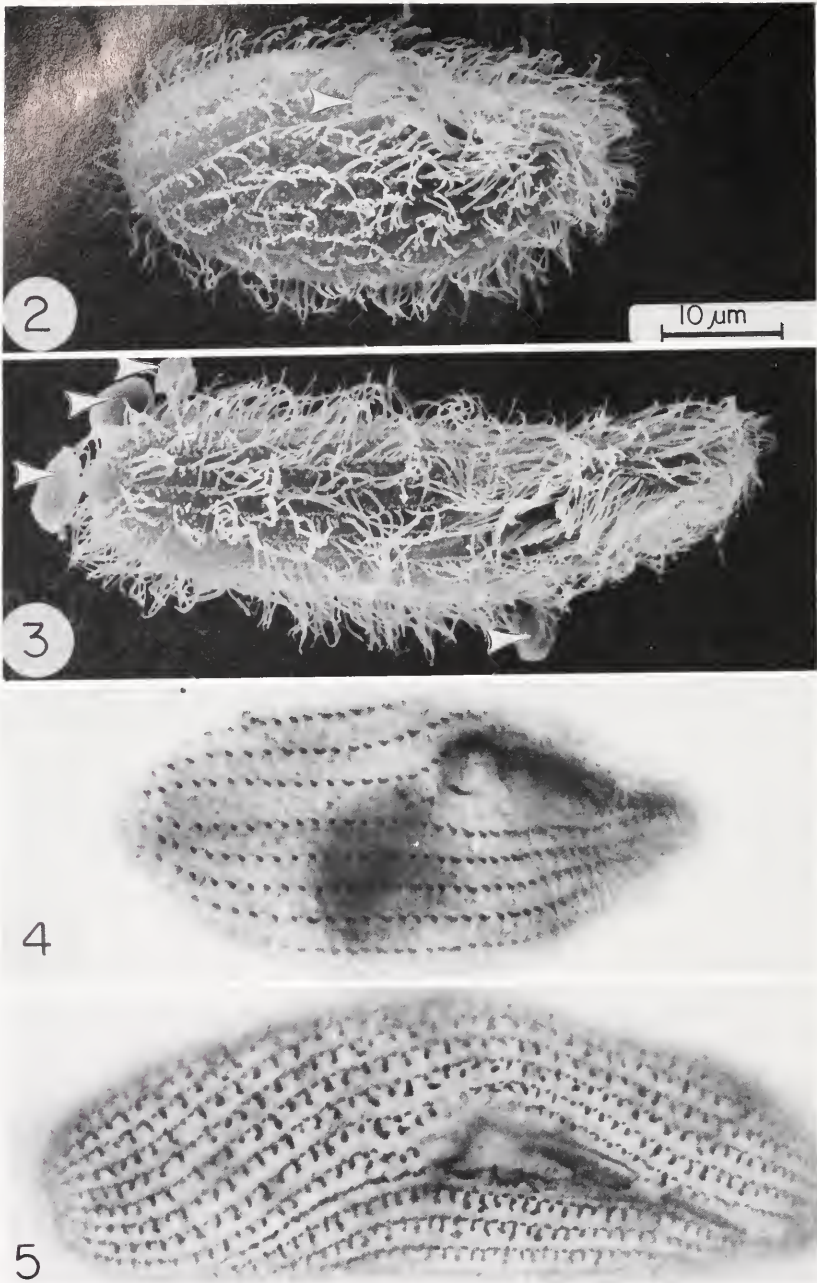


FIGURE 1. Growth curve of *Paranophrys carnivora*. Results are based on three replicate cultures.

Table III, *Artemia* homogenate and the *E. aerogenes* suspension elicited a positive chemosensory response, whereas the alga *Dunaliella parva* elicited none. These results were obtained with ciliates that had been previously cultivated on each of the diets indicated. When *Chlorococcum* sp. was offered instead of *D. parva*, the same results were obtained.

Since *Enterobacter* was routinely cultivated on brain heart agar, it was thought that its effect could have been due to the presence of some dissolved ingredients from the growth medium in the suspension. Indeed, brain heart infusion did elicit a positive chemosensory response in capillary experiments. To determine whether the bacteria themselves are effective, they were washed by centrifugation and offered to the ciliates in a capillary tube. After repeated rinsings in ASW, neither the bacterial pellet nor the supernatant gave a positive result. However, when the washed pellet was suspended in fresh ASW, incubated for up to 48 hours at 18°C, and then centrifuged again, the resulting supernatant elicited a chemosensory response. This indicates that washed bacteria excrete with time an effective substance or release such a factor upon disintegration.

Positive results were obtained with other microbiological growth media, such as proteose peptone and casein hydrolysate. In dialysis experiments with proteose peptone, *Paranophrys* was found to be attracted to those molecules which were able to pass through the cellophane membrane, e.g., amino acids. In further capillary tests to screen individual amino acids, only L-histidine, of the various amino acids tested, was as effective as betaine, a substance previously found to elicit a chemosensory response from *P. carnivora* (Kahan *et al.*, 1985). Choline and trimethylamine oxide (TMAO), compounds with a chemical structure similar to that of betaine, also elicited a positive response at least as strong as that of betaine. The four substances (beta-



FIGURES 2-5 are to the same scale and view the ventral side (note buccal cavity).

FIGURE 2. Scanning electron micrograph of trophic form of *Paranophrys carnivora* (arrowhead indicates part of an algal cell, *Dunaliella parva*, engulfed by the ciliate).

FIGURE 3. Scanning electron micrograph of swimming form of *P. carnivora* (arrowheads indicate algal cells from culture which have adhered to the ciliate).

FIGURE 4. Photomicrograph of silver-stained trophic form of *P. carnivora*.

FIGURE 5. Photomicrograph of silver-stained swimming form of *P. carnivora*.

TABLE I

Cell dimensions of the trophic and swimming forms of Paranophrys carnivora (given in micrometers)

Form	Length		Width		Range	Distance from anterior pole to end of UM $\bar{x} \pm \text{S.E.}$
	Range	$\bar{x} \pm \text{S.E.}$	Range	$\bar{x} \pm \text{S.E.}$		
Trophic	36-56 (17)	47.08 $\pm$ 1.3	18-35 (22)	25.18 $\pm$ 1.07	18-25 (17)	20.82 $\pm$ 1.17
Swimming	40-60 (18)	49.44 $\pm$ 1.47	13-22 (15)	16.67 $\pm$ 0.62	24-29 (15)	26.4 $\pm$ 0.52

Numbers in parenthesis indicate the number of observations.

ine, L-histidine, choline, and TMAO) were offered to *P. carnivora* at various concentrations; the results were analyzed as described previously. The index averaged over the experimental runs is shown in Figure 6 for each substance at the various concentrations. The average index of chemosensory response elicited by betaine, choline, and TMAO was significantly greater than 0.5, at the 5% level or more, at all the concentrations examined, and by L-histidine for concentrations of at least 10^{-3} M (concentrations of 10^{-4} , 10^{-5} , and 10^{-6} M were also tested, but the response was not significantly greater than 0.5).

Two-way analysis of variance of the index of chemosensory response showed significant effects due to material (P -value = 0.028) and concentration (P -value = 0.000). The means of the relative index of chemosensory response (the index, previously defined under Materials and Methods as "capillary tube assay," which adjusts for the level of response to the betaine standard during the same experimental run) exhibited the same behavior as the nonadjusted index, indicating that the differences in the index of chemosensory response represented in Figure 6 are not due to varying levels of overall chemosensory responsiveness (as measured by the response to the standard, betaine at 10^{-1} M) on the different days of experimental runs. The analysis of variance is based on the nonadjusted chemosensory response, as opposed to the adjusted response, because the data on the former satisfied the required statistical assumptions on the error terms more closely (see Scheffé, 1959, p. 5).

Figure 6 suggests that choline, betaine, and TMAO are similar in the response they elicit from *P. carnivora*, the main differences in the chemosensory response index being due to different concentrations. This was confirmed for betaine and choline using the S-method (Scheffé, 1959) of multiple comparisons to compare effects on the chemosensory response index due to materials or concentrations. The average index for betaine and choline at the concentration of 10^{-1} M was significantly different (at the 5% level) from the average at the 10^{-6} M level; and the differences between the averages at the 10^{-1} M and 10^{-5} M levels, and at the 10^{-2} M and 10^{-6} M levels, were almost significant at the 10% level.

DISCUSSION

Although previously thought (Czapik and Wilbert, 1986) to feed on fresh and decomposing tissues, the present study establishes that *Paranophrys carnivora* can feed on a more varied diet. This diet includes algae and bacteria in addition to tissues. In this respect it seems to be closer in its dietary spectrum to *P. thompsoni* and *P. magna* than to the other species of the genus. *P. thompsoni* was reported to live on bacteria and heterotrophic flagellates which developed in hatched gelatinous egg

TABLE II

Growth on and ingestion of different algal species by Paranophrys carnivora

Algal class: Species	Source	Algal size ^a	Ingestion of algae	Ciliates' growth ^b
Bacillariophyceae				
<i>Amphora</i> sp. 12	J.L., CUNY ^c	35–40/2–4	no	+
<i>Phaeodactylum tricornutum</i>	CMBRDG CC ^d	20–30/2.5	yes	++
Chlorophyceae				
<i>Chlamydomonas provasoli</i>	J.L., CUNY	4–8	no	+
<i>Chlamydomonas hedleyi</i>	J.L., CUNY	5–10/3–9	yes	++
<i>Chlorella stigmatophora</i>	IOLR ^e	5–6/3–4	yes	+
<i>Chlorella saccharophila</i>	IOLR	3	yes	–
<i>Chlorococcum</i> sp.	J.L., CUNY	2–3	yes	++++
<i>Dunaliella primolecta</i>	CMBRDG CC	6–14/4–13	yes	–
<i>Dunaliella</i> sp. Strain C9AA	B.G., HU ^f	10–18/8–13	no	+
<i>Dunaliella</i> sp. Strain E1	B.G., HU ^f	14–18/8–10	no	+
<i>Dunaliella</i> sp. Strain 1644	B.G., HU ^f	11–21/8–15	no	+
<i>Dunaliella</i> sp. Strain L10	B.G., HU ^f	9–14/8–13	no	+
<i>Dunaliella tertiolecta</i>	B.G., HU ^f	8–12/4–8	yes	+++
<i>Dunaliella parva</i>	B.G., HU ^f	6–12/3–8	yes	++++
<i>Dunaliella</i> sp. Strain I4	B.G., HU ^f	6–10/3–8	yes	+++
<i>Dunaliella</i> sp. Strain E4	B.G., HU ^f	5–10/3–8	yes	+++
<i>Dunaliella</i> sp. Strain Iran 6	B.G., HU ^f	5–8/3–8	yes	+++
<i>Nannochloris</i> sp. Strain W515	J.L., CUNY	8–12/6–8	no	++
Cyanophyceae				
<i>Anacystis</i> sp.	Houde ^g	2–3	yes	++
Prasinophyceae				
<i>Tetraselmis chuii</i>	IOLR	12–13/8–9	no	–
Prymnesiophyceae				
<i>Isochrysis galbana</i>	IOLR	4–6	yes	++

^a The dimensions (length/width or diameter) are given in μm .^b Rating code: +++++, excellent; +++, good; ++, fair; +, poor; –, no growth.^c John Lee, City University of New York.^d Cambridge Culture Collection.^e Institute of Oceanographic and Limnological Research, Haifa.^f B. Ginzburg, Hebrew University (Ginzburg and Ginzburg, 1985).^g E. D. Houde, University of Miami, Florida.

masses of dipterans (Didier and Wilbert, 1976), while *P. magna* was cultivated in cultures to which split peas had been added (Borror, 1972) and presumably fed on the bacterial flora. Nevertheless, comparative dietary experiments on the above-mentioned species should be further extended in order to establish their feeding pattern.

TABLE III

Chemosensory response of Paranophrys carnivora to different diets as determined by capillary assay

Diet cultivated on	Test diet offered in capillary		
	<i>Dunaliella parva</i>	<i>Enterobacter aerogenes</i>	<i>Artemia</i> homogenate
Algae (<i>Dunaliella parva</i>)	—	+	+
Bacteria (<i>Enterobacter aerogenes</i>)	—	+	+
Fresh meat (wounded <i>Artemia</i>)	—	+	+

Cultures of the ciliates were grown each on a different diet as indicated. The “+” indicates a positive response and “—”, no response.

Our attempts (unpub.) to introduce *P. carnivora* as a symbiont living in the coelenterates *Cordylophora* sp., *Cassiopea* sp., and *Aiptasia* as well as in the crustaceans *Artemia salina* and *Macrobrachium rosenbergii*, did not succeed. Two other species of the genus, *P. marina* and *P. carcini*, were found inside coelenterates (Thompson and

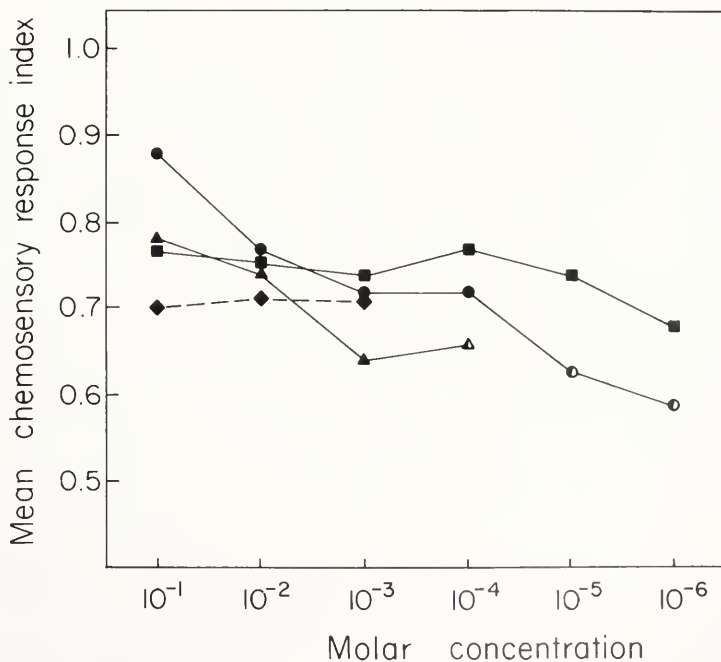


FIGURE 6. Dose-response curves for substances which elicit a positive chemosensory response from *Paranophrys carnivora* by the capillary assay. Each figure represents the mean of the values of the index of chemosensory response obtained from all of the observations for a particular substance at a certain concentration. The results obtained with betaine are indicated by circles, with trimethylamine oxide by triangles, with choline chloride by squares, and with L-histidine by diamonds. A full (black) figure indicates that the mean index of chemosensory response is significantly greater than 0.5 at the 1% level, and a half-full figure, at the 5% level. L-histidine was also tested at 10^{-4} , 10^{-5} , and 10^{-6} M, but the results were not significantly greater than 0.5.

Berger, 1965) and in the hemolymph of crustaceans (Grolière and Leglise, 1977), respectively.

Like many other ciliates (Fenchel, 1980a, b, c), *P. carnivora* ingests suspended inert particles such as polystyrene beads (unpub.) and living microorganisms. Here size seems to be a limiting factor in food ingestion. The largest food vacuoles observed did not exceed 7 μm in diameter, and algal species having size ranges above this limit were not ingested i.e., *Amphora* sp., *Dunaliella* strains C9AA, E1, 1644, L10, *Nannochloris* sp., and *Tetraselmis chuii*. However, some of the large species (the four strains of *Dunaliella* mentioned above and *Nannochloris* sp.) did sustain growth. This could be due to the ciliates' feeding on bacteria contaminating the algal cultures and/or on disintegrating algal cells in aged cultures. The same explanation could be offered for the ciliates' growth on *Phaeodactylum tricornutum*. While *C. provasoli* was in the size range of algae that could be engulfed, it was not ingested. This is probably due to the tendency of the latter algal cells to form bigger sized aggregates, or to their having a chemoinhibitory effect on phagocytosis by *Paranophrys*. Those species of algae that were ingested by *Paranophrys* (Table II) gave growth results that varied in their rating from no growth, i.e., *Chlorella saccharophila* and *Dunaliella primolecta*, to excellent growth i.e., *Dunaliella parva* and *Chlorococcum* sp. However, these latter two species did not elicit a positive chemosensory response from *Paranophrys carnivora* in our experiments. Although algae are known to release assimilated carbon into the culture medium (Hunstman, 1972; Fogg, 1977; Saks, 1982), *Dunaliella parva* and *Chlorococcum* sp. evidently do not release a substance eliciting a chemosensory response from *Paranophrys carnivora*.

Betaine, choline, L-histidine, and trimethylamine oxide, the substances found to elicit a positive chemosensory response from *Paranophrys carnivora*, are known to affect feeding behavior in various other organisms (Lindstedt, 1971; Levandowsky and Hauser, 1978; Heinen, 1980; Caprio, 1984). They are also widely distributed in various organisms including bacteria and algae (Bell and Mitchell, 1972; Levandowsky and Hauser, 1978; Edwards, 1982; Galinski and Truper, 1982; Abe, 1983; Konosu *et al.*, 1983; Shirani *et al.*, 1983; Imhoff and Rodriguez-Valera, 1984; Morihiko *et al.*, 1984) and therefore could be indicators of a wide variety of food sources for *Paranophrys carnivora*. There may be other effective substances as yet untested for *Paranophrys carnivora*, which have recently been found to elicit a chemosensory response from other ciliates such as *Paramecium* (Antipa and Norton, 1985) and *Tetrahymena* (Leick and Hellung-Larsen, 1985; Hellung-Larsen *et al.*, 1986). *Paranophrys carnivora* responds to the D-isomer of histidine, which does not occur in nature, and in this respect resembles *Tetrahymena thermophila* (Almagor *et al.*, 1981), which also responds to both the L and D forms of an amino acid (methionine).

Another characteristic *Paranophrys carnivora* shares with *Tetrahymena* is its body transformation. The morphological differences in body proportions between the ovoid feeding form and elongated swimming form of *P. carnivora* appear more pronounced when the ciliate is cultivated on tissues and on rare occasions when grown on algae, after depletion of the food organisms. A similar transformation in form appears after starvation in *Tetrahymena thermophila*. In the latter, the transformation is accompanied by several other changes i.e., oral replacement, caudal cilium appearance, and increase in number of somatic basal bodies and cilia, as well as in speed (Nelsen, 1978; Nelsen and DeBault, 1978). In *P. carnivora*, significant changes in body proportion and an increase in the somatic basal bodies have been noticed. Greater control of culture conditions of the ciliates (as may be obtained with an axenic culture) would enable further discerning and understanding of this phenomenon in *P. carnivora*.

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INTRACELLULAR pH DECREASES DURING THE *IN VITRO* INDUCTION OF THE ACROSOME REACTION IN THE SPERM OF *SICYONIA INGENTIS*

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ABSTRACT

Activation of the sperm of many invertebrate and some vertebrate species to undergo an acrosome reaction is accompanied by an increase in intracellular pH (pH_i). In each of these instances the pH_i of the unactivated cell is relatively low (6.9–7.4). Unactivated sperm of the marine shrimp, *Sicyonia ingentis*, possess an elevated pH_i (8.5). Induction of the acrosome reaction (exocytosis of the acrosomal vesicle and generation of an acrosomal filament) is accompanied by a decrease in pH_i (7.8). Low external pH elicits acrosomal filament formation in sperm that have undergone acrosomal exocytosis, but does not induce exocytosis in unreacted sperm. The ionophore, nigericin, enhances the percent of sperm that form filaments in low pH seawater ($pH < 8.0$), but does not elicit filament formation at external $pHs \geq 8.0$. Valinomycin induces filament formation in sperm that have undergone exocytosis over a wide range of external pHs (5.75–8.5). The ability of valinomycin to induce filament formation in the upper portion of this pH range (8.0) declines as the extracellular K^+ concentration rises. These results demonstrate that the sperm of *S. ingentis* undergo a pH_i decrease as a result of the acrosome reaction and that the decrease is associated with acrosomal filament formation. In addition, they also suggest that an efflux of K^+ ions is connected to the pH_i decrease.

INTRODUCTION

As a prerequisite to fertilization, most sperm must first undergo acrosomal alterations, termed the acrosome reaction (Dan, 1952). Among the majority of invertebrate sperm and sperm of a few select vertebrates, the acrosome reaction (AR) is composed of the exocytosis of the acrosomal vesicle and generation of an acrosomal filament (reviewed by Dan, 1967; Austin, 1968). The AR occurs when a sperm contacts an egg-derived inducer. The inducer, a component of one of the egg investments, interacts with the plasma membrane of the sperm and initiates a series of ionic and biochemical sperm-associated events that lead to, among other sperm-associated changes, the AR (see Shapiro and Eddy, 1980; Lopo, 1983 for reviews). For example, the ionic changes associated with the AR in the sperm of *Strongylocentrotus purpuratus* include: (1) an uptake of extracellular Ca^{++} which is thought to be involved in the exocytosis of the acrosomal vesicle (Tilney *et al.*, 1978; Shackman *et al.*, 1981); (2) a Na^+ associated H^+ efflux which is necessary for the polymerization of actin filaments

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Abbreviations: ASW (artificial sea water), DMO (^{14}C -dimethylloxazolidine 2,4-dione), EW (egg water), pH_i (intracellular pH), pH_o (extracellular pH).

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and thus, the formation of the acrosomal filament; and (3) a K^+ efflux that leads to a depolarization of the sperm membrane potential (Shackman *et al.*, 1981). Although not thoroughly documented, it appears that the ARs of many motile invertebrate sperm involve the same ionic changes.

Unlike the sperm of most invertebrates, the sperm of the natantian decapod, *Sicyonia ingentis*, are nonmotile. These cells possess an anterior spike (contained within an acrosomal vesicle), a subacrosome, and a posteriorly located main body which houses the nucleus (Kleve *et al.*, 1980; Shigekawa and Clark, 1986). *S. ingentis* sperm do not possess flagella and also lack organized mitochondria. Sperm are transferred to female seminal receptacles during mating and stored until spawning, which may occur several weeks to months later (Anderson *et al.*, 1985). Thus, these sperm remain in an unactivated state for extended periods after transfer from the male. At spawning, ova are released from paired ovopores and mixed with sperm ejected from the seminal receptacles. Sperm bind spike first to ova and become activated to undergo a biphasic AR (Clark *et al.*, 1984). Within seconds bound sperm undergo the first phase of the AR, acrosomal exocytosis (which includes the loss of the spike), and 10–20 minutes later they complete the AR by generating an acrosomal filament (second phase). Thus, *in vivo* the two phases of the AR are temporally separated. Previous work demonstrates that the first phase, acrosomal exocytosis, depends upon external Ca^{++} (Clark *et al.*, 1981) as is true in other systems. These experiments were conducted with sperm taken from males, which are not competent to form acrosomal filaments (Clark *et al.*, 1984). The ionic requirements for acrosomal filament formation have not been investigated.

The ability to induce a complete AR in sperm removed from female seminal receptacles and incubated in isolated egg products enabled us to investigate the ionic requirements of the AR's second phase, acrosomal filament formation. The present paper: (1) describes the *in vitro* induction of a complete AR using egg components; (2) presents data suggesting that the sperm possess a high intracellular pH (pH_i) prior to undergoing the AR and that a pH_i decrease is associated with the second phase of the AR (formation of the acrosomal filament); and (3) provides data indicating that the outward movement of K^+ ions is involved in the pH_i drop.

MATERIALS AND METHODS

Collection and maintenance of animals

Specimens of *Sicyonia ingentis* were collected using an otter-trawl in 60–100 meters of water off San Pedro, California. Live animals were transported in chilled seawater (8–10°C) to the Bodega Marine Laboratory and maintained in flow-through seawater tanks at ambient temperatures (10–14°C). Gravid females were isolated and kept under constant light in a 500 gallon flow-through tank. The lights were turned off to initiate spawning. Animals were monitored for spawning under a red light (Kodak Wratten #2 filter).

Collection of egg water

Spawning animals were removed from the tank and held over 50 ml glass beakers containing chilled (4°C) artificial seawater (ASW) prepared according to Cavanaugh (1956). After the negatively buoyant ova ($1-2.5 \times 10^3$) had settled to the bottom of the beaker, approximately $\frac{3}{4}$ of the seawater was drawn off. The ova were then resuspended by swirling and kept in suspension for five minutes. The remaining ASW

containing egg-derived components was then pipetted out of the beakers, cleared by centrifugation ($100,000 \times g$, 15 min), and divided into 1 ml aliquots. The protein concentration of each egg water (EW) batch was determined after the method of Lowry *et al.* (1951). EW was stored in liquid nitrogen if not used immediately.

Collection of sperm

In *S. ingentis*, only sperm that have been transferred to a female and stored in the female's seminal receptacles are competent to: (1) undergo the acrosome reaction (AR) in response to egg derived components; and (2) form an acrosomal filament as part of the AR, regardless of the manner of induction (Clark *et al.*, 1984). As a result, only sperm taken from seminal receptacles of the female were used. Seminal receptacles from ten or more females were pooled, homogenized in ASW using a Wheaton 5 ml tissue grinder to free sperm, and hand centrifuged to remove fragments of empty receptacles. Free sperm were pelleted from the supernatant at $200 \times g$ for five minutes. Pelleted sperm were resuspended in ASW and used within one hour of isolation.

Induction of the acrosome reaction with egg water

Isolated sperm (10^6 cells) were incubated in 1 ml of experimental (containing EW) and control (containing ASW) solutions. Aliquots of cells in each experiment were fixed (with a drop of 5% glutaraldehyde in ASW) at appropriate times and scored with phase microscopy ($400\times$) for: (1) percent unreacted; (2) percent that had undergone acrosomal exocytosis but had not formed acrosomal filaments; and (3) percent fully reacted (sperm which possessed acrosomal filaments). For each experimental run (n), duplicate 20 μl aliquots were removed and 100 sperm were scored in each for acrosomal status.

Intracellular pH determinations

Isolated sperm (2.1×10^8) were divided into three equal samples. One sample was incubated for 10 min in 1.6 ml of ASW (pH 8.0); this sample was used to measure the pH_i of unreacted sperm. A second sample was incubated in 1.6 ml of EW (pH 7.8) for 10 min; these sperm were used to measure the pH_i of sperm that had undergone acrosomal exocytosis. The last sample was incubated in 1.6 ml of EW for 50 min; these sperm were used to measure the pH_i of fully reacted sperm. Intracellular pH determinations were made with ^{14}C -dimethylloxazolidine 2,4-dione (DMO) using a modification of the technique described by Waddell and Butler (1959). At the conclusion of the initial incubations, each of the three samples was divided in half: (1) 25 μl of $^3\text{H}_2\text{O}$ and 50 μl of ^{14}C inulin were added to one; and (2) to the other, 25 μl of $^3\text{H}_2\text{O}$ inulin and 50 μl of ^{14}C DMO (final concentration of 33 μM) were added. After a 20 min equilibration period: (1) triplicate 20 μl aliquots (controls) were transferred to scintillation vials containing 15 ml of ACS scintillation fluid (Beckman); (2) 10 μl samples were removed, fixed, and scored for acrosomal status; and (3) triplicate 200 μl samples were microfuged (Fisher Model #235B) for 90 seconds through a 95 volume percent silicone oil (Dow Corning 704)–5 volume percent hexane solution, the supernatants were removed, and the tips of the microfuge tubes (containing the sperm pellets) were cut off and placed in scintillation fluid. The samples sat overnight and were then counted on a Beckman LS100 scintillation counter. Calculation of internal water space and pH_i determinations followed those described by Shackman *et al.* (1981).

pH induction experiments

Sperm ($10^7/\text{ml}$) were incubated for 5 min in either ASW pH 8.0 or EW pH 8.0 after which 100 μl aliquots were added to 900 μl of ASW at the following pHs: 5.75, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. Reactions were halted 10 min later with a drop of 5% glutaraldehyde (in ASW) and acrosomal status was scored. In experiments using sperm that had spontaneously undergone acrosomal exocytosis in ASW alone, sperm were scored until 100 reacted cells had been observed in each duplicate. In those using EW to induce acrosomal exocytosis, counts were performed as described above. The pH of ASW was determined on an Orion (EA920) pH meter. Above pH 8.0, ASW was adjusted with 0.1–1.0 *N* NaOH; below pH 8.0 it was adjusted with 0.1–1.0 *N* HCl or 0.2 *M* acetate buffer. All ASWs were pH adjusted just prior to use.

Ionophore induction experiments

In separate experiments, sperm ($10^7/\text{ml}$) were induced to undergo acrosomal exocytosis in EW as described above and 100 μl aliquots were added to 900 μl of ASW at the pHs described in the previous section. Immediately after the addition of sperm, 5 μl of nigericin (0.5 *mM* in 100% DMSO) or valinomycin (1.0 *mM* in 100% DMSO) were added, with mixing, to the sperm suspensions. Control samples contained 0.5% DMSO. Aliquots of each treatment were fixed with a drop of 5% glutaraldehyde (in ASW) 5 minutes after the addition of the ionophores or DMSO and sperm were scored for acrosomal status. In addition, samples were removed and fixed to determine levels of acrosomal exocytosis prior to introduction into pH ASWs and the addition of the ionophores.

ASWs of different $[\text{K}^+]$ were obtained by adding or deleting equal molar amounts of KCl and NaCl from the MBL formula for ASW (Cavanaugh, 1956).

RESULTS

Induction of the acrosome reaction by egg water

Acrosomal status of *S. ingentis* sperm is easily scored with phase microscopy. Figure 1 illustrates an unreacted sperm, a sperm that has undergone acrosomal exocytosis, and a fully reacted sperm (possessing an acrosomal filament). Sperm removed from the seminal receptacles of females and incubated in 50 $\mu\text{g}/\text{ml}$ (protein) of egg water (EW) undergo a complete AR (acrosomal exocytosis and formation of an acrosomal filament) in which the temporal separation between the two phases is maintained. Within 1 min of exposure to EW, *S. ingentis* sperm underwent acrosomal exocytosis at levels greater than ASW controls (41% as compared to 7.3%) and by 5 min, greater than 75% of the sperm had undergone acrosomal exocytosis (Fig. 2). These sperm (after a 15 min incubation in EW) had only undergone acrosomal exocytosis; they did not possess acrosomal filaments. Sperm that had undergone acrosomal exocytosis did begin to form filaments, commencing approximately 30 min after introduction into EW (Fig. 3). At 45 min, more than 50% of all sperm counted possessed acrosomal filaments; this translates into more than two-thirds of exocytosed sperm possessing filaments. By 60 min, approximately 60% of all sperm (85% of exocytosed sperm) had formed filaments. The sperm that formed acrosomal filaments were those that had undergone acrosomal exocytosis. The total percent of sperm that had either undergone only exocytosis or undergone a complete AR remained constant through 60 min (Fig. 3).

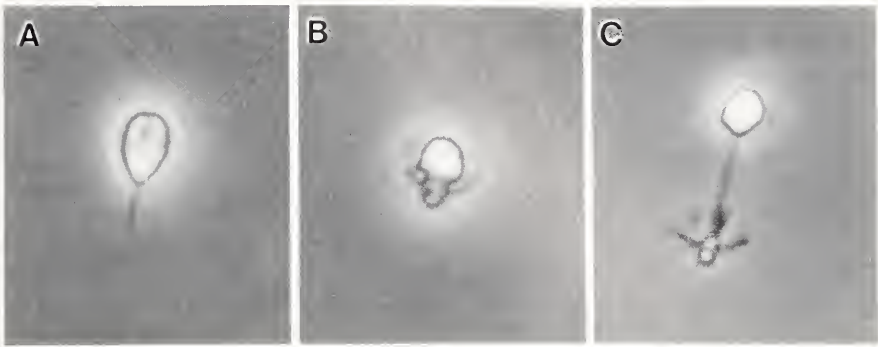


FIGURE 1. Phase micrographs of the three activation states of *Sicyonia ingentis* sperm; (A) an unreacted sperm possessing an anterior spike, (B) a sperm that has undergone acrosomal exocytosis and has lost the spike, and (C) a fully reacted sperm possessing an acrosomal filament.

A small but consistent number of sperm (8–10%) isolated in artificial seawater (ASW), and not transferred to EW, underwent acrosomal exocytosis (Fig. 2). This percent not only remained constant with increased incubation times, but exocytosis was the only portion of the AR that occurred. Sperm isolated to ASW have been observed for up to 180 min without seeing acrosomal filaments.

Intracellular pH measurements

Based on the accumulation ratios of the DMO uptake experiments we have calculated an average pH_i for unreacted and reacted *S. ingentis* sperm. Unreacted sperm

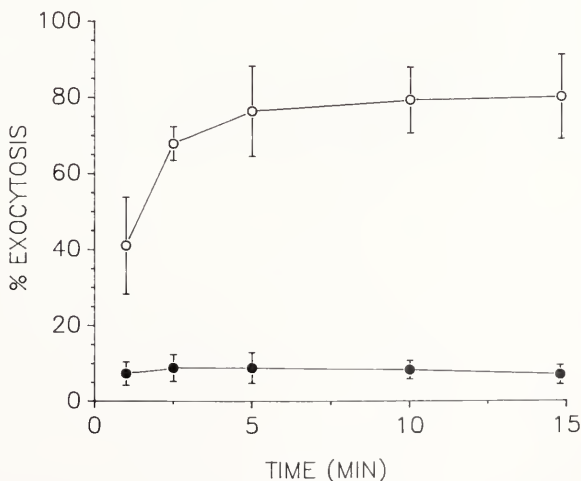


FIGURE 2. EW induction of acrosomal exocytosis; response over time. Sperm were incubated in 50 $\mu\text{g}/\text{ml}$ EW (○) or ASW (●), fixed at the times designated above, and scored for acrosomal status. Data points are means; vertical lines are standard deviations ($n = 4$). Each replicate utilized a separate batch of sperm and EW.

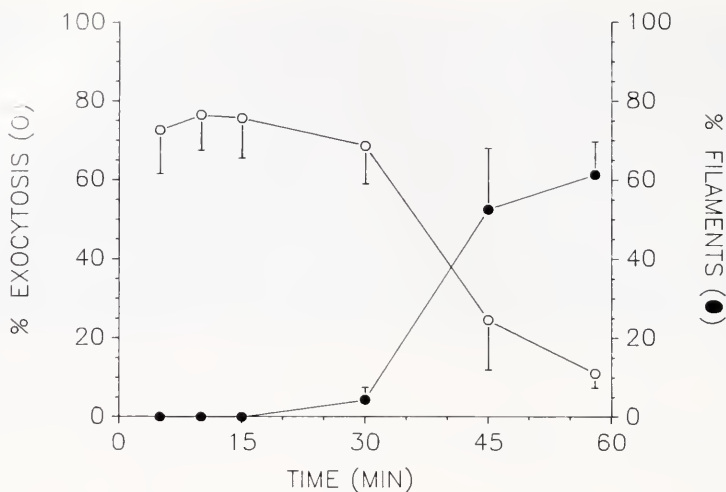


FIGURE 3. Acrosomal filament formation as a function of time. Sperm were incubated in 50 $\mu\text{g}/\text{ml}$ of EW. Aliquots of sperm were removed, fixed, and scored for percent exocytosis (○) and percent formed filaments (●) at each time point. Data points are the means of four replicates; each replicate was conducted with different batches of sperm and EW. Vertical lines are standard deviations.

removed from seminal receptacles and placed in ASW (pH 8.0) possessed a pH_i of 8.47 ± 0.27 . In these samples, greater than 91% of the sperm were unreacted and none of the reacted sperm possessed acrosomal filaments (Table I). Sperm incubated in EW (pH 7.8) for 10 min prior to the addition of the DMO possessed a significantly lower pH_i of 7.81 ± 0.13 ($P < 0.05$). Greater than 74% of these sperm had undergone acrosomal exocytosis and approximately 3% possessed acrosomal filaments at the end of the equilibration period (30 min after sperm had been introduced into EW). The pH_i of sperm incubated in EW for 50 min, 8.01 ± 0.06 , was also lower than that of unreacted sperm ($P < 0.05$), but was not significantly different from the 10 min EW samples. Seventy-one percent of the sperm incubated in EW for 50 min possessed filaments at the end of the equilibration period (70 min after introduction into EW).

TABLE I

pH_i of *Sicyonia ingentis* sperm

Sample	Exocytosed ¹	Filaments ²	pH_i
ASW	8.3 ± 2.5	0	8.47 ± 0.27
EW ₁₀	72.3 ± 3.1	2.7 ± 1.5	7.81 ± 0.13
EW ₅₀	6.0 ± 2.0	71.0 ± 2.0	8.01 ± 0.06

Isolated sperm were reacted in EW for 10 min (EW₁₀) and 50 min (EW₅₀) and used to measure pH_i of sperm that had undergone only acrosomal exocytosis and sperm that had fully reacted, respectively. The pH_i of unreacted sperm (ASW) was measured after incubating isolated sperm for 10 min in ASW.

¹ Percent of sperm which had undergone acrosomal exocytosis only at the time of disruption.

² Percent of sperm which had undergone a complete AR at the time of disruption.

Effect of external pH on the AR

External pH (pH₀), within the range examined, does not elicit the first phase of the AR, acrosomal exocytosis, in *S. ingentis* sperm (Fig. 4A). The percentages of sperm that were unreacted after transfer to the pH ASWs did not vary significantly with pH₀. This was true for those sperm that had been preincubated in ASW alone, as well as for those sperm that had been preincubated in EW. In those experiments where sperm had been preincubated in EW, the percent that did not undergo acrosomal exocytosis averaged 26.1 for all pH₀s with no observable pH-dependent trend. The same held true for those sperm that were not exposed to EW (incubated in ASW only); the percent of these sperm that did not react averaged 90.7 for all pH₀ treatments. Such was not the case with regard to acrosomal filament formation.

Exposure of sperm to low pH₀ did elicit the formation of acrosomal filaments. Sperm induced to undergo exocytosis with EW and subsequently transferred to pH₀s of less than 7 underwent filament formation within 10 min of transfer (Fig. 4B). This represents a reduction of the temporal separation between the two phases of 20–35 min. The pH₀ optimum for filament induction was between pH 5.75 and 6.5. Below pH 5.75, exocytosed sperm were disrupted and above pH 7.0, sperm did not form acrosomal filaments within the reduced temporal window. Similar results were obtained with sperm that had spontaneously undergone exocytosis in ASW (not incubated in EW). The percentages of these sperm that formed filaments after exposure to low pH₀ were somewhat less than the sperm treated with EW, but the effect of pH₀ was similar (Fig. 4).

Induction of acrosomal filament formation by ionophores

The response of sperm to low pH₀ was enhanced with the addition of either nigericin or valinomycin, however, the pH₀ optima were different for both ionophores (Fig. 5). Greater than 50% of sperm that had undergone acrosomal exocytosis in EW and were subsequently exposed to nigericin for 5 min underwent filament formation in pH₀ 6.0–7.5. At pH₀ 5.75, $31.5 \pm 8.2\%$ of such sperm possessed filaments, however, the number of unreacted sperm was twice that of the other pH₀ treatments, suggesting that at pH₀ 5.75 reacted sperm were disrupting (Fig. 5A). With the addition of nigericin, not only were the percentages of filament formations increased at low pH₀s (6.0–7.5), but the range for filament induction was shifted 0.5–1.0 units basic.

Valinomycin not only elicited more filament formations than any of the other treatments, but it was also effective over a broader range of pH₀s than the other treatments (Fig. 5). Greater than 80% of exocytosed sperm underwent filament formation in pH₀ 5.75–8.0. At pH₀s 9.0 and 9.5 the percentages of filaments were dramatically reduced. As in low pH₀ inductions in the absence of ionophores, there was no observable effect on acrosomal exocytosis (Fig. 5A).

The ability of the ionophore valinomycin to induce formation of acrosomal filaments was pH₀ dependent when extracellular K⁺ was elevated (Fig. 6). Filament formation in pH 6.0 ASW was not reduced by increasing concentrations of extracellular K⁺, however, a steady decline in the percentage of filaments was observed in pH 8.0 ASW as the K⁺ level was increased. When the [K⁺] was increased from 10 mM to 20 mM, filament formation at pH₀ 8.0 decreased by approximately 50%. At 30 mM K⁺ filament formation declined by another 50%, and at 40 mM K⁺ no filaments were observed. In pH 9.0 ASW, filaments were only formed in low K⁺ (K⁺-free ASW).

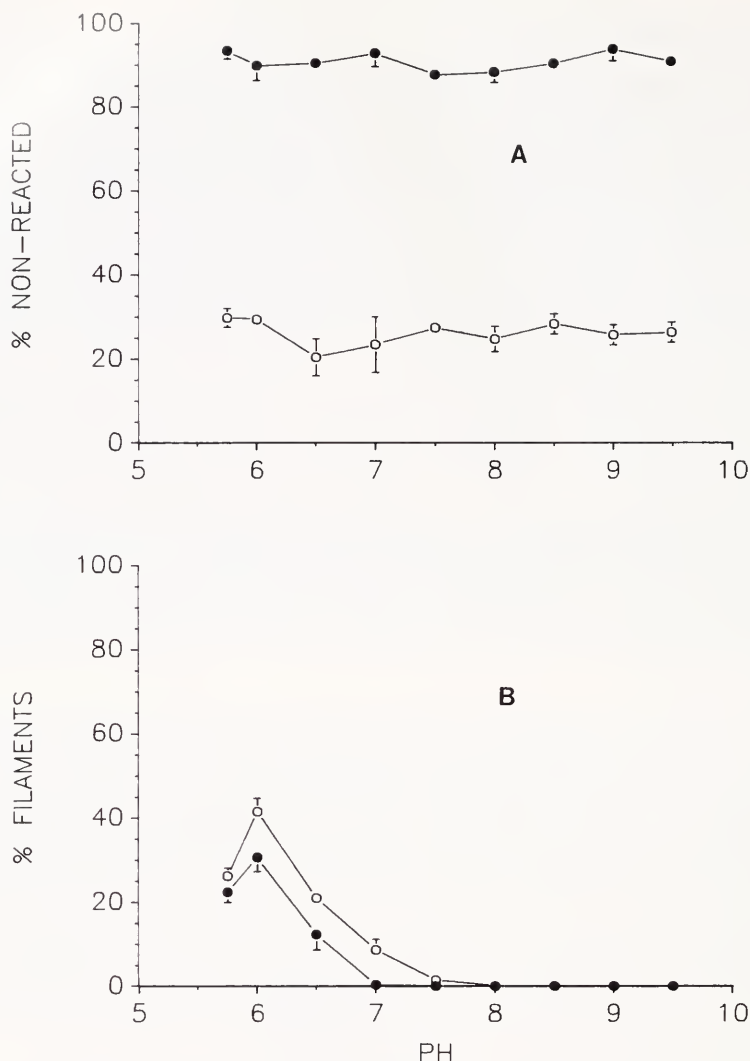


FIGURE 4. Effects of external pH upon the AR. (A) Acrosomal exocytosis. (B) Acrosomal filament formation. Sperm ($10^7/\text{ml}$) were incubated for 5 min in either ASW pH 8.0 (●) or EW pH 8.0 (○) after which $100\ \mu\text{l}$ aliquots were added to $900\ \mu\text{l}$ of ASW at the pHs indicated above. Reactions were halted 10 min later with a drop of 5% glutaraldehyde (in ASW) and acrosomal status was scored. Data points in (B) represent mean % of exocytosed sperm that formed filaments.

DISCUSSION

Induction of the two phases (acrosomal exocytosis and acrosomal filament formation) of the AR in *S. ingentis* sperm is temporally separated and sequential *in vivo* (Clark *et al.*, 1984). Upon binding to ova, sperm undergo acrosomal exocytosis and some 10–20 min later undergo acrosomal filament formation. The present report has demonstrated that the *in vitro* induction of this AR in sperm removed from female

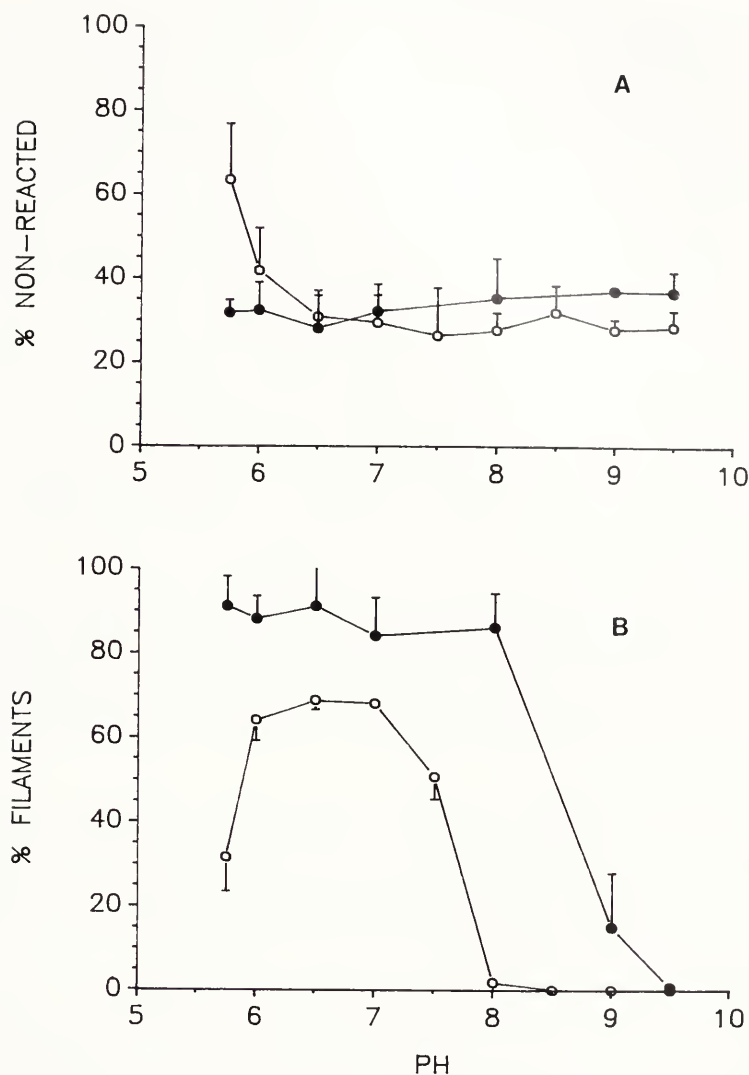


FIGURE 5. The effects of pH_0 on valinomycin and nigericin induction of the AR. (A) Acrosomal exocytosis. (B) Acrosomal filament formation. Sperm ($10^7/\text{ml}$) were first induced to undergo acrosomal exocytosis in EW. 100 μl aliquots were added to 900 μl of ASW at the pHs indicated above, and then exposed to either 1 μM nigericin (○) or 5 μM valinomycin (●). Sperm were fixed after 5 min and scored for reactions. Data points in (B) represent mean percent of exocytosed sperm that formed acrosomal filaments; vertical lines are standard deviations. $n = 3$. Each n in each experimental batch represents sperm pooled from different females, different ASWs, and different EW and nigericin solutions.

seminal receptacles and incubated in solutions containing isolated egg components (EW) is also temporally separated and sequential. *In vitro*, acrosomal exocytosis is achieved within 2.5–5 min, yet sperm that have undergone acrosomal exocytosis do not form acrosomal filaments for an additional 30–45 min. Thus the temporal separation that is observed on the surface of an ovum is preserved albeit lengthened under

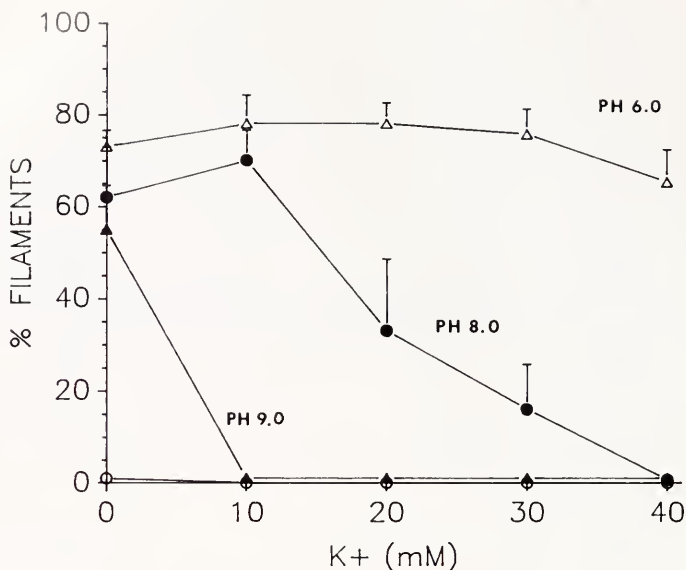


FIGURE 6. Acrosomal filament formation and external K^+ . Sperm ($10^7/\text{ml}$) were induced to undergo acrosomal exocytosis in EW (5 min). One hundred ($100\ \mu\text{l}$) samples were then transferred to $900\ \mu\text{l}$ of ASWs containing from 0 to $40\ \text{mM}$ K^+ at pH_0 6.0 (Δ), pH_0 8.0 ($\bullet$), and pH_0 9.0 ($\blacktriangle$). Samples were fixed at 5 min and scored for acrosomal status. Control samples ($\circ$) were preincubated in ASW (not exposed to EW), added to ASW (pH 8.0) containing the described $[K^+]$, and exposed to 0.5% DMSO. Data points are mean percent of exocytosed sperm that formed filaments ($n = 3$); vertical lines are standard deviations.

in vitro conditions. The ability to elicit a complete AR *in vitro* and the fact that the two phases are separated has allowed the dissection of the two phases with respect to the controls of activation. Based upon direct measurements of pH_i , low pH_0 inductions of the AR, and the effects of both low pH_0 and external $[K^+]$ on ionophore inductions, we propose that formation of the acrosomal filament in *S. ingentis* sperm is associated with a pH_i decrease.

Measurements of intracellular pH in *S. ingentis* sperm suggest that: (1) unreacted sperm possess a high intracellular pH; (2) prior to formation of the acrosomal filament these cells undergo a pH_i decrease; and (3) subsequent to filament formation they do not return to the unactivated pH_i . Although DMO is a widely used probe for determining pH_i , it does have limitations (Roos and Boron, 1981; Busa and Nuccitelli, 1984). These include: (1) DMO measurements reflect an average pH for the cell and do not provide information on the pH of subcellular compartments (e.g., the acrosomal vesicle or the subacrosome); and (2) alkaline membrane-bound organelles can sequester DMO, giving an erroneous picture of the pH of other subcellular compartments (Roos and Boron, 1981; Busa and Nuccitelli, 1984). For example, Grinstein *et al.* (1984) have reported that a DMO measured pH_i increase at lymphocyte proliferation is in fact not an activation pH_i change, but rather an increase in the number of mitochondria (which results in an increased DMO uptake by the cells). The structural organization and the direction of the measured pH_i change in *S. ingentis* sperm, however, allowed us to entertain the supposition that the pH_i change was real and was associated with filament formation. Unreacted *S. ingentis* sperm possess three subcellular regions: a nucleus, a subacrosome, and an acrosomal vesicle;

mature sperm do not possess mitochondria (Shigekawa and Clark, 1986). As a result, any pH_i changes would be expected to be associated with one of these compartments and two of them are involved in the AR. We would not expect, *a priori*, an overall pH_i decrease to occur simply as a result of acrosomal exocytosis; the acrosomal vesicle is an acidic organelle (Kleive *et al.*, 1980) and therefore its loss at exocytosis might be expected to yield an increase in average pH_i . It was therefore reasonable to expect the pH_i changes to be associated with the subacrosome.

Results of the low pH_0 induction experiments correlate well with the pH_i measurements and delineate at which phase of the AR the pH_i drop occurs. Neither low pH (≤ 7.5) alone nor low pH in conjunction with nigericin or valinomycin induce unreacted sperm to undergo acrosomal exocytosis. All three do induce acrosomal filament formation in sperm that have undergone exocytosis. It follows that the pH_i decrease is associated with the second phase of the AR, formation of the acrosomal filament. Furthermore, low pH_0 elicits filament formation in sperm that have exocytosed in ASW and have not been exposed to EW. This indicates that the pH_0 is not acting through a pH alteration of EW, rather, it is directly influencing filament formation. These observations are in contrast to previous studies demonstrating that a net rise in pH_i occurs during the AR in sperm of other species (Shackman *et al.*, 1981; Working and Meizel, 1983; Matsui *et al.*, 1986). By contrast with the sperm of *S. ingentis*, these cells have been reported to possess depressed pH_i s prior to activation. For example, the pH_i of unreacted *S. purpuratus* sperm is between 6.6 and 7.3, based upon measurements obtained with weak bases (Shackman *et al.*, 1981). Using 9-aminoacridine, the pH_i of the sperm of the starfish *Asterias amurensis* and *A. pectinifera* was reported to be 7.4–7.5 (Matsui *et al.*, 1986). In the hamster sperm, the intracrosomal pH has been measured to ≤ 5 , also using 9-aminoacridine (Meizel and Deamer, 1978).

The ionophore nigericin exchanges K^+ or Na^+ for H^+ (the selectivity for K^+ over Na^+ is more than an order of magnitude), thus it is an electroneutral ionophore that dissipates proton gradients across cell membranes (Pressman, 1976; Johnson and Scarpa, 1976). As such, the pH_i of sperm in the presence of nigericin should more closely parallel the pH_0 of the ASW than in the low pH_0 experiments conducted without ionophore. The results of the nigericin induction experiments agree well with the measured pH_i changes that occur during the AR. Based on the DMO measurements, sperm decrease pH_i from 8.5 to between 7.8–8.0 as a result of the AR. Nigericin elicits filament formation at pH_0 s 6.0–8.0 in sperm that have undergone exocytosis. Since the pH_0/pH_i gradient at pH_0 s above 8.0 would not favor a nigericin induced pH_i decrease, filament formation would not be expected. Conversely, as the pH_0 is decreased, it would be expected that at some pH_0 an acid overload in the presence of nigericin would occur. This occurs between pH 5.75 and 6.0 in *S. ingentis* sperm.

Valinomycin, like low pH ASW and nigericin, does not elicit acrosomal exocytosis, but will induce acrosomal filament formation. However, unlike the other two, valinomycin is pH -independent over a wide pH_0 range (pH 5.75–8.0) at normal extracellular K^+ concentrations (10 mM). The ability of valinomycin to elicit filament formation does become sensitive to pH_0 at elevated extracellular K^+ concentrations. In 10 mM K^+ ASW filament formation proceeds at pH_0 6.0 and 8.0; no filaments are seen at pH_0 9.0. As the $[\text{K}^+]$ is increased to 40 mM (in 10 mM increments), filament formation declines approximately 50% at each incremental rise in $[\text{K}^+]$ in pH 8.0 ASW. At pH_0 9.0, filament formation is inhibited in $[\text{K}^+] \geq 10$ mM; however, filament formation will proceed if the K^+ concentration is below 10 mM. Valinomy-

cin transports only K^+ (the selectivity over Na^+ is greater than three orders of magnitude) across membranes and therefore is electrogenic (Johnson and Scarpa, 1976; Pressman, 1976). The results of the valinomycin/pH/ K^+ experiments suggest that the ionophore is facilitating a K^+ efflux, however, they also suggest that the K^+ efflux does not in itself elicit filament formation. The fact that filament formation in pH 8.0 ASW is very sensitive to small changes in the extracellular K^+ concentration leads us to suggest that the pH_i decrease elicits acrosomal filament formation. The ionophore facilitates a K^+ efflux which results in an alteration of the sperm membrane potential (hyperpolarization?) and this change in membrane potential drives a proton influx.

This study has demonstrated that unactivated sperm of *S. ingentis* possess an unusually high resting pH_i , that they undergo a decrease in pH_i as a result of the AR, and that the pH_i decrease is associated with formation of the acrosomal filament. The pH_i measurements and shifts that occur during the AR in *S. ingentis* sperm must be viewed within the context of this unique system. These cells, after transfer to a female, are stored for several weeks or more in exoskeletal seminal receptacles during which time they undergo maturational and/or capacitational changes (Clark *et al.*, 1984). During storage they are separated from the seawater (pH *ca.* 8.0–8.2) by only the seminal plasm in which they are embedded. Thus, these cells probably maintain a pH_i in the same region as that found in their environment (seawater). At least two possibilities arise that would functionally explain why these sperm possess such a high unactivated pH_i : (1) the energetic costs of maintaining an elevated pH_i are less than if pH_i were depressed below physiological levels (*ca.* 7.0–8.0); or (2) since sperm undergo maturational/capacitational changes while in the seminal receptacles of the female, the elevated pH_i might be associated with these processes (*e.g.*, in the prevention of premature filament formation). These, of course, are not all inclusive nor are they mutually exclusive; rather, they are questions that await investigation.

ACKNOWLEDGMENTS

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A MORPHOLOGICAL EXAMINATION OF GASTRULATION IN A MARINE ATHECATE HYDROZOAN

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ABSTRACT

The early embryonic development of the marine hydrozoan *Halocordyl disticha* is examined via light histology and transmission electron microscopy. Particular emphasis is devoted to the gastrula and the mode of gastrulation. Cleavage in *Halocordyl disticha* is irregular, total, and asynchronous resulting in the production of stereoblastulae. Each stereoblastula forms a blastopore at the future posterior end of the larva and gastrulates via invagination to produce a lecithotrophic planula larva. During gastrulation spherical surface cells radially migrate toward the blastopore, become cuboidal-shaped in the region of the pore, and disappear to the interior of the embryo. Gastrulation requires 2 h to complete, during which time the ectoderm becomes separated from the endoderm by a mesoglea, interstitial cells arise in the central endoderm, and the embryo elongates to form a planula larva. This study presents the first documented example of invagination in the Hydrozoa.

INTRODUCTION

Cnidarians represent an early phase of metazoan evolution. Their simple architecture combined with their exceptional morphogenetic plasticity and adaptability make them popular animals for examining developmental processes and principles. The phylum is unusual in that its postembryonic development has been more thoroughly investigated than its embryogenesis. This is surprising because the cnidarians offer excellent material for the study of the evolution of embryogenesis. In the simpler cnidarians embryogenesis may appear "anarchic," whereas in the more advanced forms one sees complex mosaic patterns of embryogenesis (Metschnikoff, 1886; Carre, 1969).

Since the work of Metschnikoff in the late 1800's a few papers dealing with embryonic development of the Hydrozoa have been published (Van de Vyver, 1964, 1967, 1980; Bodo and Bouillon, 1968; Mergner, 1972; Freeman, 1981; Martin and Archer, 1986). Mergner (1972) attempted to provide a general overview of the processes involved in cleavage, germ layer formation, and postembryonic development and concluded that there was great diversity in hydrozoan developmental modes. Van de Vyver (1980) analyzed via light histology modes of cleavage, germ layer formation, and postembryonic development of several species of hydrozoans and concluded that the modes of embryonic development in the Hydrozoa are restricted to a very few which are commonly distributed in the animal kingdom. She stated that two types of cleavage commonly occur in the Hydrozoa and that cleavage is dependent upon yolk quantity of the egg. As a general rule, cleavage for eggs adequately supplied with yolk is radial, total, and adequal. Such cleavage is characteristic of eggs of Filifera or

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Capitata corynoidea developing in a gonophore or spawned into the water by free-swimming medusae. Large yolk-filled eggs such as those of Capitata tubularoidea undergo irregular cleavage. Van de Vyver (1980) further concluded that the most important difference between the types of cleavage in the hydrozoans is the presence or absence of a blastocoele since its occurrence will determine the mode of germ layer formation. Metschnikoff (1886) proposed that eggs released by free-swimming medusae form coeloblastulae while others developing inside gonophores form stereoblastulae. Van de Vyver (1980) suggested that although Metschnikoff's first point might be true, his second is certainly not. Furthermore, Van de Vyver (1980) stated that within the Hydrozoa the processes of gastrulation are numerous and may vary from species to species. Within the Hydrozoa gastrulation has been shown to occur via either ingression, multipolar ingression, delamination, or simple cellular rearrangements (Jägersten, 1972; Tardent, 1978; Van de Vyver, 1980). Invagination has not yet been reported in the Hydrozoa although it is common in anthozoans and scyphozoans (Tardent, 1978; Van de Vyver, 1980). Van de Vyver (1980) reported that polar ingression, multipolar ingression, and delamination are characteristic of coeloblastulae, whereas in stereoblastulae the cells which occupy the periphery of the embryo simply become progressively different from those situated in the center. She claimed that no movements of cells occur in stereoblastulae.

From the above discussion it is quite clear that our basic knowledge concerning embryonic morphogenesis in the cnidarians is sketchy and that additional studies of embryogenesis in this phylum are needed. In this study the early development of a marine athecate hydrozoan, *Halocordyl disticha*, is analyzed via light histology and transmission electron microscopy. Particular emphasis is devoted to the gastrula. *Halocordyl disticha* is a member of the suborder Capitata and forms free-swimming medusae which release eggs and sperm into seawater where fertilization is external. Cleavage is irregular, asynchronous, and total resulting in the formation of stereoblastulae which gastrulate via invagination to produce lecithotrophic planula larvae. This study presents the first documented example of invagination in the Hydrozoa.

MATERIALS AND METHODS

Mature colonies of the marine hydrozoan *Halocordyl disticha* were collected from pier pilings at the University of North Carolina Institute of Marine Sciences in Morehead City, North Carolina. Fronds from mature male and female colonies were placed together in large finger bowls of filtered seawater. Subsequently, the bowls were placed in the dark at 6:00 pm and returned to the light at 9:00 pm. Within 1 hour after exposure to light early cleavage stages were found in the bottoms of the dishes. These embryos were transferred to small finger bowls of filtered seawater and reared at 23°C to the planula stage.

Early cleavage embryos, late cleavage embryos, blastulae, gastrulae, and young planulae were prepared for either light histology or transmission electron microscopy. Animals for light microscopy were fixed for 1 hour in 10% formalin in seawater, dehydrated in an ethanol series, and embedded in Paraplast Plus paraffin. Serial sections, 10 μ m thick, were mounted on glass slides and stained with either Azure B or the Schiff's reagent (nuclear feulgen reaction). Live embryos and prepared histological sections were photographed with a Zeiss standard research microscope. Embryos undergoing cleavage and gastrulation were also continuously examined under the microscope until young planulae were formed.

Samples for electron microscopy were fixed for 1 h in 2.5% glutaraldehyde, pH

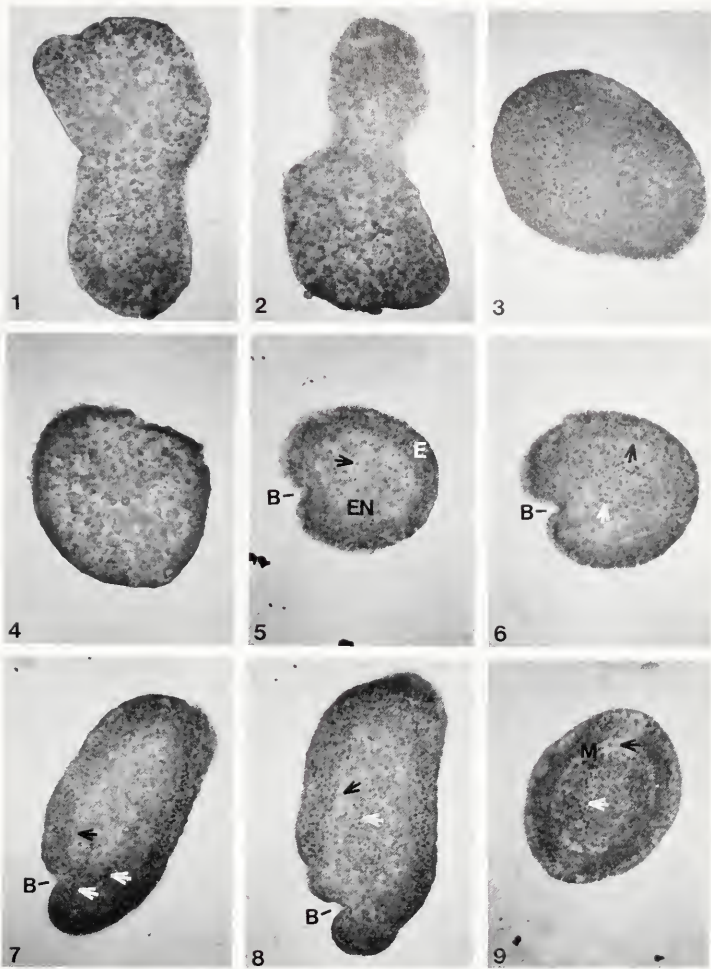
7.4, in 0.2 M phosphate buffer. They were postfixed for 1 h in 2% osmium tetroxide, pH 7.2, in 1.25% sodium bicarbonate. Specimens were dehydrated in an ethanol series, infiltrated, and embedded in Spurr's embedding media. Blocks were serially sectioned on a Porter-Blum MT-2B ultramicrotome, placed on 150-mesh copper grids, and stained with 3.5% uranyl acetate in ethanol followed by lead hydroxide. Grids were examined and photographed with a Hitachi H-600 transmission electron microscope.

Surface cells of live embryos at various stages of gastrulation were marked with Nile Blue and subsequently monitored for their movement. The marking technique involved using a 0.01% solution of Nile Blue in seawater. The dye was drawn into microcapillary pipettes with varying bore diameters. Embryos were immobilized for marking by placing them in a tiny groove in the bottom of a Falcon small plastic petri dish. The dye-containing micropipettes were gently touched to the surfaces of the embryos for 30–60 seconds producing small blue patches of marked cells of varying diameter (depending upon pipette bore size) along the animal surface. Previous marking studies using planulae indicate that Nile Blue is nontoxic at the 0.01% concentration and embryos stained with Nile Blue retain the dye for several days. The dye will not diffuse into unstained tissue (Martin, unpub.). After marking the gastrula cells, half of the animals were removed from the grooves and returned to small dishes containing filtered seawater. The other half were left immobilized in the grooves and their dishes placed in a moist chamber to prevent samples from drying. The marked cells of the immobilized and free-moving animals were continuously examined throughout gastrulation for change in axial position.

RESULTS

Cleavage in embryos of *Halocordyl disticha* is holoblastic, unequal, and asynchronous (Figs. 1–4). Cleavage begins 1 h after fertilization and results in the formation of blastomeres of unequal size. A period of early cleavage extends to the beginning of 6 h postfertilization during which time no one embryo cleaves in exactly the same fashion. Such embryos assume numerous bizarre shapes and sizes and reach the 128–256 cell stage (Martin and Archer, 1986). Early cleavage is rapid and by 6 to 8 h postfertilization a stereoblastula (late cleavage) is formed (Figs. 3, 4). The stereoblastula assumes the shape of a sphere and the blastomeres are more uniform in size than during early cleavage. The stereoblastula is ca. 230 μm in diameter and consists of an outer layer of small spherical blastomeres surrounding an inner layer of larger spherical blastomeres (Fig. 4).

By 8 h postfertilization the surface of the embryo is smooth and a single indentation appears at one pole of the embryo (Figs. 5, 6, 10–12). This indentation corresponds to a blastopore, and the pole at which it forms marks the future posterior pole of the planula (Figs. 7, 8, 13, 14). This stage represents gastrulation and the young gastrula is ca. 250 μm long and 190 μm wide (Fig. 11). Gastrulation requires 2 hours to complete and during this time a number of events occur (Figs. 5–20). The initial blastopore indentation will deepen to form a groove (Figs. 5, 6, 10–12). Some of the cells on the surface migrate in a radial fashion toward the deepening blastopore, invaginate over the lips of the pore, and disappear to the inside. Such movement of cells is easily visualized using the Nile Blue marking procedure. Marked patches of blue cells move toward the blastopore, briefly inhabit the lips of the pore, and eventually disappear from the surface of the animal. Hence on a marked animal a blue patch of cells can be traced from the surface to the blastopore region, and ultimately to the



FIGURES 1-9. Histological sections of developing embryos of *Halocordyl disticha*.

FIGURE 1. Early cleavage embryo (3 h postfertilization) $\times 200$.

FIGURE 2. Early cleavage embryo (3 h postfertilization) $\times 200$.

FIGURE 3. Late cleavage 7-h embryo (stereoblastula). Note the absence of a mesoglea and interstitial cells. $\times 200$.

FIGURE 4. Stereoblastula (7 h postfertilization) $\times 200$.

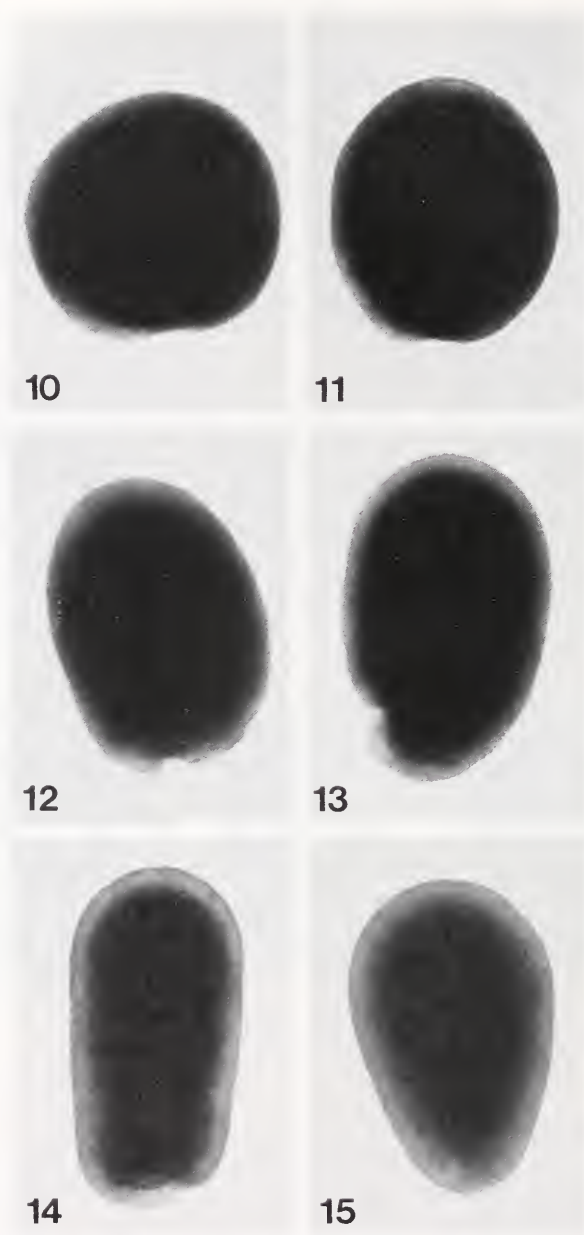
FIGURE 5. Early 8-h gastrula. An early blastopore (B) is visible. Separation of the two germ layers is apparent and interstitial cells (arrow) appear in the central endoderm. E, ectoderm; EN, endoderm. $\times 200$.

FIGURE 6. Mid-gastrula stage. The blastopore (B) has deepened to form a groove. A mesoglea (black arrow) is seen as are numerous interstitial cells (white arrow). $\times 200$.

FIGURE 7. Nine-hour gastrula which has begun to elongate. The blastopore (B) is located at the posterior pole of the embryo and a mesoglea is present (black arrow). Cells migrating over the lips of the blastopore to the inside are visible (white arrows). $\times 200$.

FIGURE 8. Elongating 9-h gastrula. Central endodermal interstitial cells (white arrow) are distinguishable from the outer endodermal/gastrodermal cells (black arrow). B, blastopore. $\times 200$.

FIGURE 9. Ten-hour planula. The ectoderm is separated from the endoderm by an acellular mesoglea (M). White arrow, interstitial cells; Black arrow, gastrodermal cells. $\times 200$.



FIGURES 10-15. Gastrulation in *Halocordyl disticha*.

FIGURE 10. Early 8-h gastrula with a slight indentation (blastopore) at the future posterior pole. $\times 75$.

FIGURE 11. Mid-8-h gastrula with a deepening blastopore. $\times 75$.

FIGURE 12. Late 8-h gastrula with a prominent blastopore. Lips of the blastopore are visible and the embryo has begun to elongate. $\times 75$.

FIGURE 13. Elongating 9-h gastrula. The embryo has a distinct anterior end and a posterior end. The blastopore is visible at the posterior end. $\times 75$.

FIGURE 14. Late 9-10-h gastrula which has elongated. The blastopore is still visible at the posterior pole. $\times 75$.

FIGURE 15. Ten-hour planula. The blastopore has completely closed producing a 2 germ layered planula larva. $\times 75$.

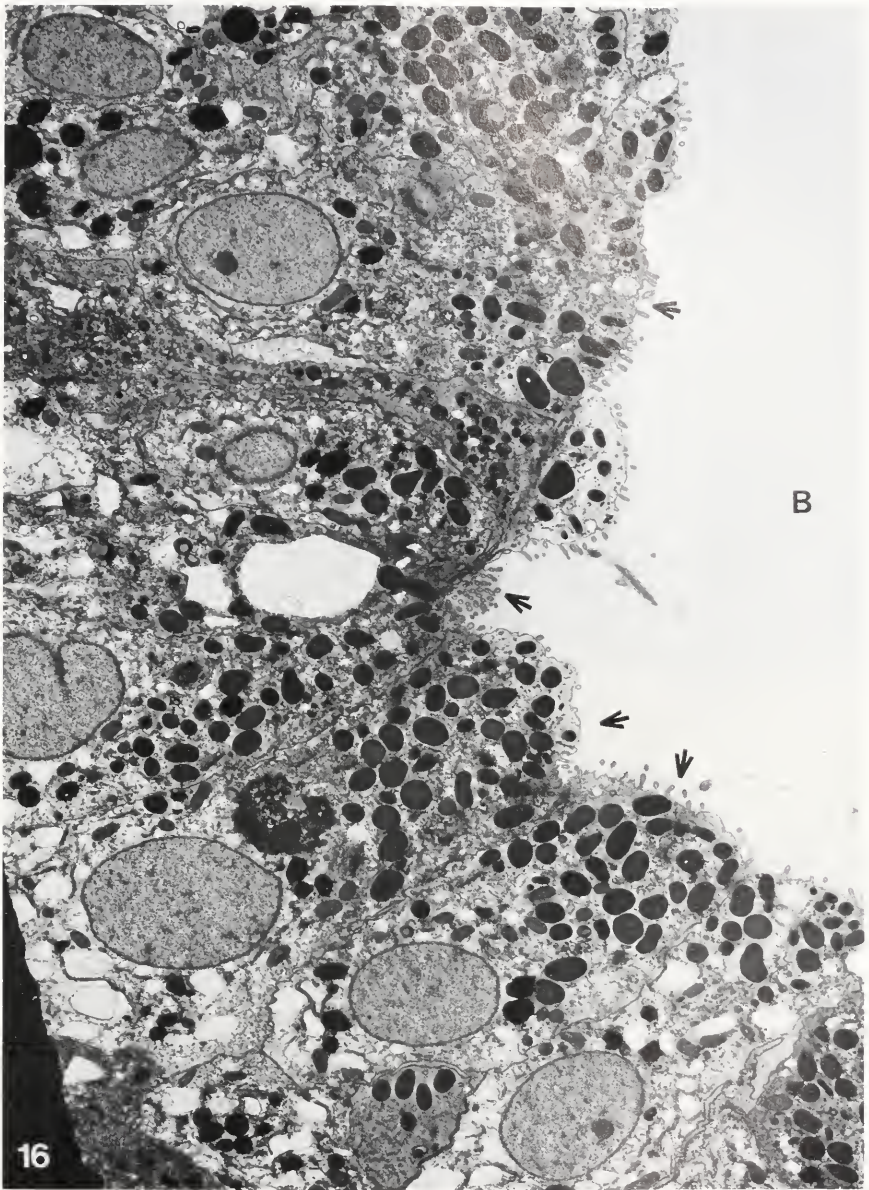


FIGURE 16. Longitudinal section through the blastopore region of an 8-h embryo. As cells move into the region of the pore they change from a spherical shape to a cuboidal shape (arrows). Microvilli and cilia from cells forming the lips of the blastopore project into the space of the pore (B). $\times 4900$.

animal interior. Time required for such patch movement (*i.e.*, from the initial marked surface position to the disappearance at the blastopore) varies anywhere from 15–30 minutes. The shape of the migrating cells changes as they move inward.

Examination of the blastopore region via transmission electron microscopy illus-

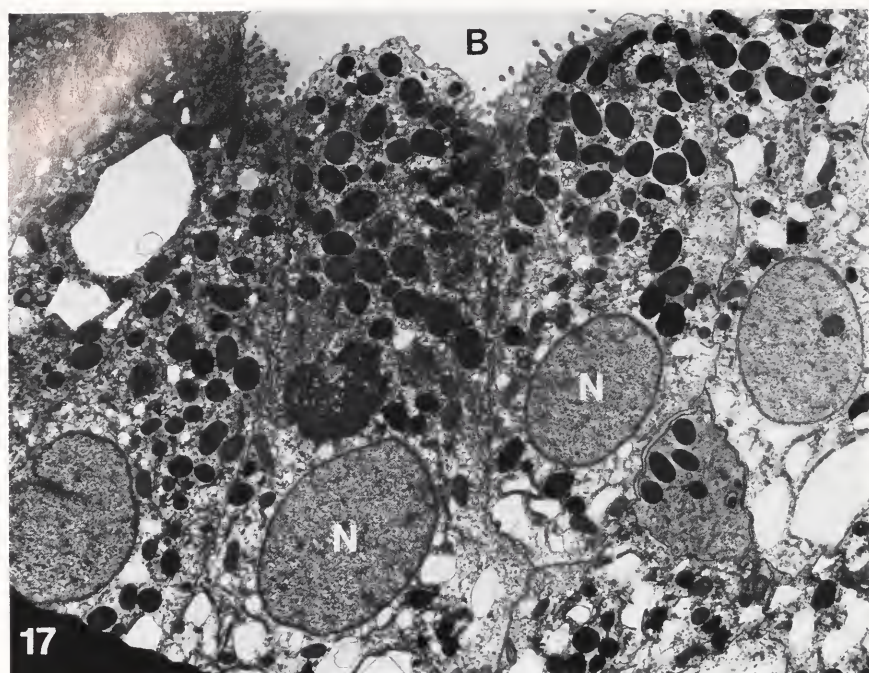


FIGURE 17. Enlargement of a portion of the blastopore region of an 8-h embryo. Cuboidal-shaped cells form the lips of the pore. B, groove of the blastopore; N, nuclei of cells in the blastopore area. $\times 5000$.

trates the true nature of the indentation (Figs. 16–18). In the region of the blastopore groove, spherical surface cells become cuboidal (Figs. 16, 17). Hence the cells forming the lips of the blastopore are cuboidal. Such cuboidal-shaped cells possess cilia and microvilli that project into the groove of the pore (Figs. 16–18). The cuboidal cells of the blastopore eventually disappear to the interior of the embryo. As cells invaginate a clear separation of the ectoderm and endoderm becomes visible with the formation of an acellular mesoglea (Figs. 5–8).

During gastrulation there is localization of embryonic tissue types within the endoderm. The presumptive gastrodermal cells become distinguishable from the mesenchymal-like interstitial cells (Figs. 5–8, 19, 20). The interstitial cells appear as an aggregate of cells in the central endodermal core of the embryo during invagination. At this time some cytodifferentiation has begun since interstitial cells stain more darkly with azure B than do the more peripheral gastrodermal cells (Fig. 8). In the early gastrula (just prior to blastopore formation) the central blastomeres consist of large yolk-filled masses (Fig. 19). Such blastomeres appear to be loosely packed in the center of the embryo as indicated by the large intercellular spaces between the blastomeres (Fig. 19). At this stage interstitial cells are not yet present. Once invagination begins the loose arrangement of the central blastomeres is lost (intercellular spaces disappear) and clusters of small round interstitial cells appear in the center of the embryo (Fig. 20). Such interstitial cells become clearly segregated from the outer forming columnar gastrodermal cells.

Between 8 and 10 h postfertilization the gastrula elongates in an anterior-posterior

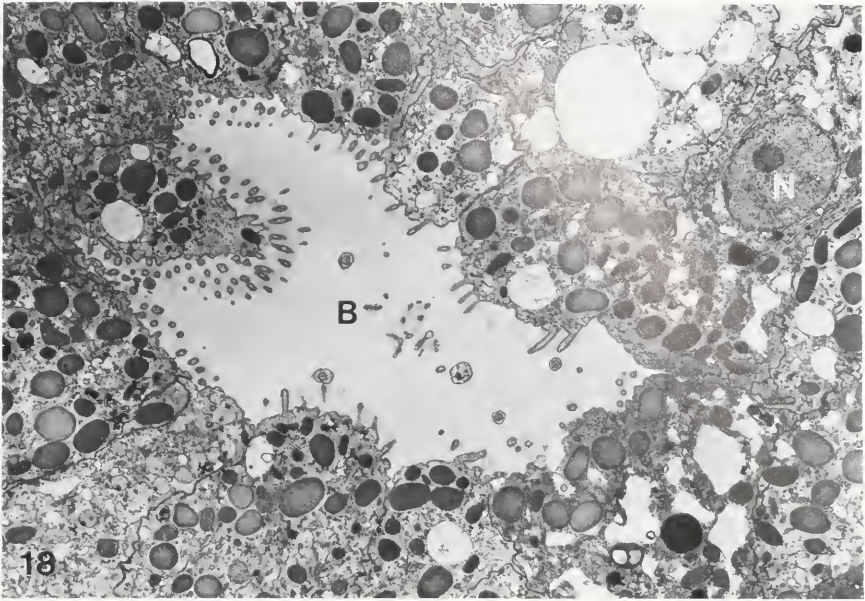


FIGURE 18. Cross-section through the blastopore region of an 8-h embryo. Microvilli and cilia of migrating cells extend into the space of the blastopore (B). N, nucleus of cell in region of the blastopore. $\times 6700$.

direction to form a young planula (Figs. 7-9, 12-15). The 10-h planula is *ca.* $350\ \mu\text{m}$ long, $180\ \mu\text{m}$ wide in the anterior region, $170\ \mu\text{m}$ wide in the mid region, and $120\ \mu\text{m}$ wide in the tail (Fig. 15). By 10 h the planula has a distinct anterior end and posterior end. The blastopore is located at the posterior pole of the planula and will soon close (Figs. 14, 15). No gastrovascular cavity or mouth is found in the planula at any stage of its development. The 10-h planula will elongate to form a mature planula (24-96 h old depending on temperature) which will attach via its anterior end to a substrate and undergo metamorphosis.

DISCUSSION

Within the Cnidaria the processes of gastrulation are numerous and vary widely from species to species (Tardent, 1978). Among the anthozoans gastrulation has been reported to occur via either invagination, delamination, multipolar ingression, or a combination of invagination and polar ingression (Tardent, 1978). In scyphozoans gastrulation may occur via invagination, polar ingression, multipolar ingression, or invagination plus polar immigration (Tardent, 1978). In hydrozoans examples of gastrulation by polar ingression, multipolar ingression, and delamination have been reported. However, until now no examples of invagination have been documented (Jägersten, 1972; Tardent, 1978).

Jägersten (1972) provided a brief overview of gastrulation in the cnidarians and stated that within the phylum a connection existed between the mode of gastrulation and whether the formed larva was lecithotrophic or planktotrophic. In species which gastrulate via either delamination, multipolar ingression, or unipolar ingression, the

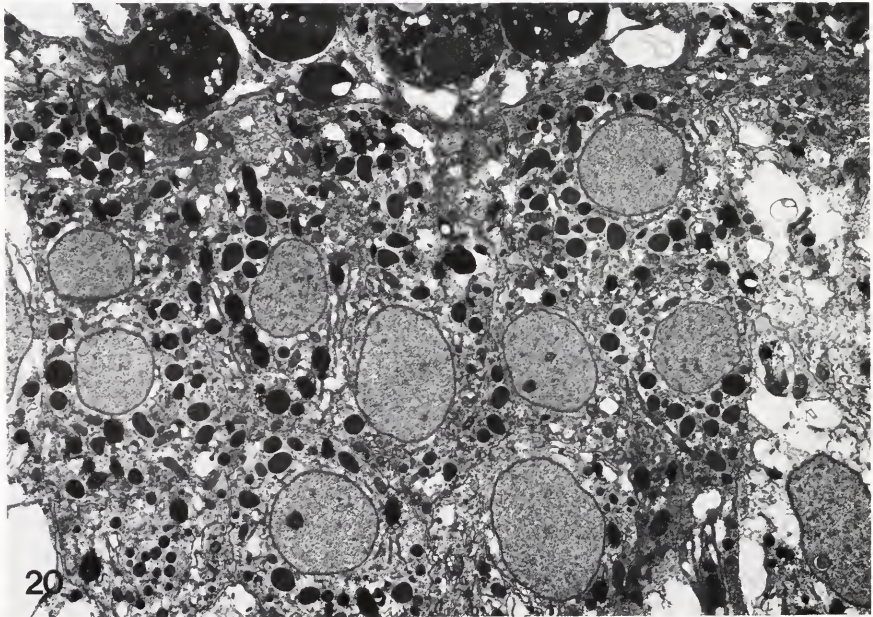
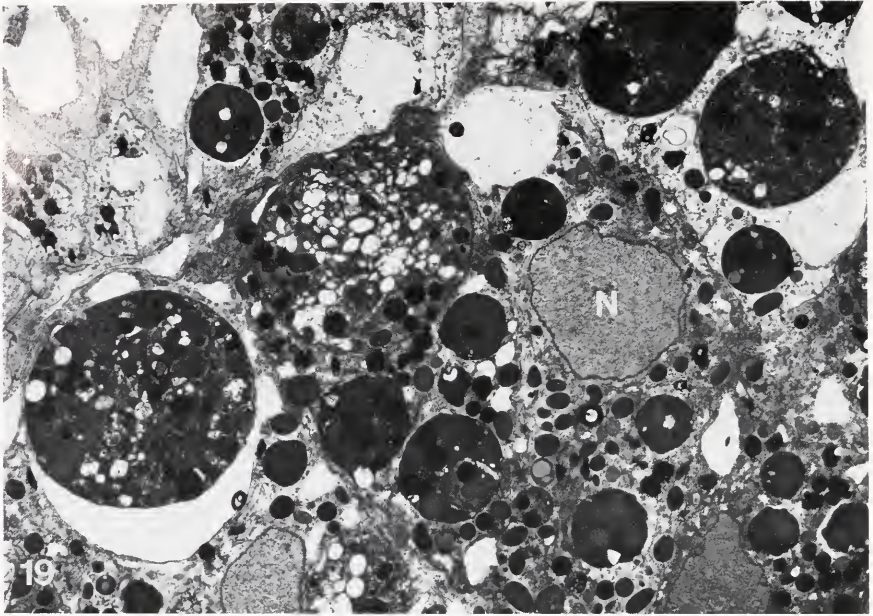


FIGURE 19. Central inner blastomeres of an early 8-h embryo. These central endoblast cells are filled with yolk and are separated from each other by large intercellular spaces. No distinguishable interstitial cells are yet present. N, nucleus of central endoblast cell. $\times 4000$.

FIGURE 20. Central endoblast region of a 9-h embryo. Clusters of young interstitial cells are visible. As the interstitial cells increase in number the intercellular space decreases and the central region of the embryo assumes a more compact appearance. The interstitial cells are completely set apart from the outer gastrodermal cells during gastrulation. $\times 4000$.

derived larvae exhibit lecithotrophy and never planktotrophy. In species which produce actively feeding larvae (planktotrophic) the mode of gastrulation is via invagination. Jägersten (1955, 1959) presented arguments supporting the ideas that the original method of gastrulation in the cnidarians was via invagination, that the planktotrophic larval life was the primitive condition, and that lecithotrophy was a secondary trait which arose independently on different occasions within the phylum. Furthermore he stated that lecithotrophy is dominant among the Cnidaria. Jägersten (1972) and Widersten (1968) proposed that the most primitive features of the phylum are found within anthozoans and the most altered within the hydrozoans. Jägersten (1972) further said that lecithotrophy may occur in larvae which exhibit invagination (e.g., *Pachyercinathus*). Despite the moderate quantity of yolk in the eggs of these animals, invagination persists.

This study documents the occurrence of invagination in the Hydrozoa. Embryos of *Halocordyl disticha* form stereoblastulae which gastrulate via invagination to produce lecithotrophic planula larvae. Marking studies clearly indicate that surface cells migrate to the blastopore, occupy the lips of the pore, and eventually disappear to the interior of the gastrula. Neither a mouth nor a gastrovascular cavity form in these planulae. The absence of a mouth in cnidarian embryos which gastrulate via invagination is not uncommon, as examples also exist among the scyphozoans (*Aurelia*, *Cyanea*) (Jägersten, 1972).

Jägersten (1972) claimed that the common ancestor of the Metazoa included a Gastrea form, a creature with both an alimentary cavity and a mouth. He proposed that the almost universal distribution of the invagination gastrula was conclusive evidence for the Gastrea theory. The hydrozoans can now be added to this universal list as invagination gastrulae are found within this class. Furthermore, if Widersten (1968) and Jägersten (1972) are correct in their assumptions that invagination is the primitive condition within the Cnidaria and that the anthozoans are the more primitive class, then the invagination process as described in this paper for a marine hydrozoan may illustrate a stubborn retention of this original primitive condition. Clearly, further investigations of early embryogenesis in the Hydrozoa concentrating on modes of gastrulation are needed to complement the work presented for *Halocordyl disticha*.

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VARIABILITY IN THE PATTERN OF SEXUAL REPRODUCTION OF THE CORAL *STYLOPHORA PISTILLATA* AT EILAT, RED SEA: A LONG-TERM STUDY

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ABSTRACT

Sexual reproduction of the Red Sea coral *Stylophora pistillata* was followed at Eilat in a long-term study (1974–1984). Field examination of over 9000 colonies through 119 months indicated that *S. pistillata* had a reproductive season of approximately 8 months (varying from 6 to 9 months). Premature planulae and eggs were aborted following winter storms, resulting in a lowering of the planular index and the number of female gonads per polyp. Histological examinations of tissue from 20 large colonies which were studied for several years, until they were found dead *in situ*, indicated that either sexuality (reproductive states) and/or fecundity could be completely altered from one reproductive season to the next: *i.e.*, hermaphroditic colonies exhibiting high fecundity in one season became male or even sterile thereafter, and vice versa. In addition, great variability in reproduction between successive years was recorded in sexuality and in the fecundity of shallow water populations. Shallow water colonies (5 m) possessed up to 5 times more female gonads per polyp and shed 5 to 20 times more planulae than deep water colonies (25 to 45 m) in which the reproductive season is 2 to 3 months shorter.

We suggest that the changes in the hermaphroditic, male, or sterile modes of reproduction in *S. pistillata* are from energy limitations and stress conditions. Since reproductive activity probably involves significant energetic expenditures, any stress or diminution in energy resources affects sexuality or fecundity. This should be considered before formulating any general hypothesis on coral reproduction.

INTRODUCTION

Much information concerning reproductive biology of scleractinian corals has recently become available. Fadlallah (1983) reviewed past studies and provided a list of almost 90 species in which several known reproductive characteristics are presented. More recent studies (Harriott, 1983; Szmant-Froelich *et al.*, 1984; Shlesinger and Loya, 1985; Wallace, 1985; Willis *et al.*, 1985; Babcock *et al.*, 1986; Szmant, 1986) provide information on reproductive patterns of more than 100 additional species of corals.

Although this list of studied species is impressive, data on scleractinian reproduction is still scanty, especially that dealing with their reproductive ecology. These studies evaluated sizes, shapes, and numbers of gonads, and attempted to establish reproductive seasonality, lunar periodicity, mode of reproduction, planula characterizations, and behavior. However, most studies were based on observations and

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experiments carried out within a period of a year or less. Only a few studies dealt with longer periods ranging from two (Atoda, 1947a, b; Harriott, 1983; Jokiel, 1985; Wallace, 1985) to three years (Kojis and Quinn, 1981a; van Moorsel, 1983; Stoddard and Black, 1985). Consequently, studies on sexual reproduction often fall short in documenting many aspects of coral reproduction (Fadlallah, 1983). Detailed information on coral reproduction could clarify many aspects of their life history patterns and provide a better understanding of the coral reef as a whole.

Stylophora pistillata (Esper) is one of the most abundant coral species in the Gulf of Eilat, Red Sea. Some aspects of the reproduction of this species have already been studied in the field and the laboratory. Descriptions of planulae and gonads have been made (Rinkevich and Loya, 1979a). In addition, synchronization in breeding, colony size in relation to fecundity, onset of reproduction, reproduction within a single colony, and seasonality of planulation were also reported (Rinkevich and Loya, 1979b). This paper summarizes results of a ten-year study on the reproduction of *S. pistillata* which elucidate some general conclusions characterizing coral reproductive activities.

MATERIALS AND METHODS

Reproductive activity of *S. pistillata* was studied from March 1974 to January 1984 (most intensively from 1976 to 1980). The study area was located in front of the H. Steinitz, Marine Biological Laboratory at Eilat, Gulf of Eilat, Red Sea, and was visited regularly once a month during the ten-year study period. Large colonies (mean geometric radius, $\bar{r} > 20$ cm) were sampled from both shallow (3–5 m) and deep water (25–60 m) populations.

Reproduction was studied by two techniques: collections of shed planulae (see below) and examinations of gonads in histological sections (Rinkevich and Loya, 1979a, b). A single branch was sampled from each colony. This branch represents the reproductive state of the entire colony (Rinkevich and Loya, 1979b). The number of female gonads was counted within serial sections for each tested polyp and quantitative data were obtained on the average number of eggs per polyp in a given specimen (6–18 polyps per sample). Male gonads were not counted because of the difficulty of following them in serial histological sections as a result of the irregular shape of a typical male gonad (Rinkevich and Loya, 1979a). Tissue samples were always taken near the bases of branches since few polyps from the tips contain genital cells (Rinkevich and Loya, 1979b; Kojis and Quinn, 1981a).

Early in the study, planulae were collected *in situ* by covering large colonies with plankton nets in the late afternoon and removing the nets at midnight (Rinkevich and Loya, 1979a). However, due to the difficulties with this technique during night diving (especially with the deep-water colonies), planulae were collected from coral branches that were brought into the laboratory. The branches were carefully removed underwater using wire cutters, and transported to the laboratory within 30 min after sampling in closed, separate plastic bags. The water in each bag was checked for the appearance of planulae. Each sample was put separately in a 5 l glass aquarium, containing filtered seawater, and left overnight. Planulae were shed during the night (Rinkevich and Loya, 1979a). Although handling stimulated planula release, it is assumed that these planulae were prepared for shedding. This assumption was supported by the finding that the released planulae were fully developed. Since conditions in all treated samples were similar, we concluded that collection procedures did not affect the results. Planulae were counted by sight and removed by pipettes. The seawater was then filtered through a plankton net (100 μ m) and all remaining planulae were

TABLE I

Some characterizations of recorded southern storms in Eilat

Date	No. of storms	Waves		Winds	
		Max. height (m)	Max. length (m)	General direction	Max. speed (km/h)
Feb. 1979	3	2	12	SE	nd
Apr. 1979	3	1+	nd	S	nd
Nov. 1979	1	1	6	SE	20
Dec. 1979	2	2	nd	SE	nd
Feb. 1980	2	2.5	10	SSE	35
Mar. 1980	1	nd	nd	nd	nd
Apr. 1980	1	nd	nd	nd	nd
Jan. 1981	2	1.5	20	S, SE, SW	25
Feb. 1981	3	1.2	27	SSE	18
Mar. 1981	1	0.5	nd	S, SE	nd

nd = no available data. (Personal communication, C. Porter, Israel Oceanographic and Limnological Research Ltd., Eilat).

collected and counted. Sampled branches were placed on a filter paper for 15 min to remove excess water and weighed (accuracy to the nearest 1 g). In most cases branch weights ranged between 100–200 g. Results are presented as number of released planulae per 100 g of coral skeleton, during one night.

In addition, the release of planulae from mature colonies ($\bar{r} > 20$ cm) was checked each month *in situ* where several branches were carefully broken from many colonies. This procedure stimulated the release of planulae in colonies which were in a reproductive state. The shed planulae were easily seen and traced by sight underwater. A planular index was then formulated (see below), which took into account the relative number of planulae shed and the percentage of reproducing colonies. Since variability in the fecundity between different colonies within the population was high (Rinkevich and Loya, 1979b), up to 90 large colonies were sampled each month (in 2–3 replicates, at least 30 colonies in each) to assess the validity of the planular index. The index sign (–) was given when none of the sampled colonies released any planulae; (+–) when very few planulae were released (total number of 1–5 planulae from the 30 tested colonies in each replicate); (+) when about one third of the colonies released few planulae; (++) when up to two thirds of the colonies shed planulae (many or few; many = any small fragment broken from the colony released about two planulae); (+++) when most or all of the colonies shed planulae.

The most severe storms in the sea at Eilat are known as southern storms, which occur during the winter and spring. Some physical parameters of these storms are partly documented from February 1979 (Table I).

RESULTS

Long-term study on seasonality of planulae shedding

Planulation in *S. pistillata* was continuously studied between March 1974 to January 1984 by sampling more than 9000 colonies (Table II). The two questions investigated were whether planulation occurs in the same months from one year to the next and how the planular index in the shallow water population fluctuated during the

TABLE II

Monthly planular index in shallow water populations of Stylophora pistillata during 119 months of observations

Year	Planular index* in											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
1974			+++	++	++	+	-	-	-	-	-	+
1975	+	++	+++	+++	+++	++	+-	-	-	-	+-	+
1976	++	+++	+++	+++	+++	++	-	-	-	-	-	-
1977	-	++	+++	+++	+++	++	+	-	-	-	-	+
1978	+	++	+++	+++	++	+	+-	-	-	-	-	-
1979	++	++	++	+++	+++	+	-	-	-	-	-	+-
1980	+	+-	+++	++	+++	++	+	-	-	-	-	+
1981	+	+	+++	+++	++	++	+-	-	-	-	-	+-
1982	++	+++	+++	+++	+++	++	-	-	-	-	-	-
1983	+	+++	+++	+++	+++	+++	+	-	-	-	-	-
1984	-											

* (-), No planulae; (+-), very few; (+), few; (++) intermediate; (+++), large numbers.

119 months of observations. *S. pistillata* has a long reproductive season (planulae release) lasting approximately 8 months, from December to July (Table II). However, the reproductive season ranged 6 months (in 1976) to 9 months (in 1975). In the three-month period from August to October, no planulation was ever recorded. Only once during the ten-year investigation were very few planulae observed in November (in 1975). A marked variation was noted in the December-January-February index between different years. Although these months represent the beginning of the reproductive season (Rinkevich and Loya, 1979b), this variability might also be the result of the southern storms which are most severe during the winter (Table I). This phenomenon is also demonstrated in another part of the present study: in April 1980 a southern storm interrupted our field sampling. Branch samples were collected before the storm from 10 mature colonies. Nine of them released high numbers of planulae (average of 30 ± 28 planulae, per 100 g skeleton, per colony). One day after the storm samples were collected for histological study from 13 other mature colonies inhabiting the same area and depth. Only eight colonies contained low numbers of female gonads while the others were either sterile or contained only male gonads. The average number of female gonads per polyp, per colony was very low (0.4 ± 0.6), much lower than other April months (for more detail, see Tables VI and IV, respectively).

Long-term study on reproductive states

Two separate sets of experiments followed the long-term state of reproduction in shallow water populations. In the first experiment 20 large colonies ($\bar{r} > 20$ cm) were chosen (December 1976) and sampled for histological study two to three times a year *e.g.*, in the beginning, the peak and the end of the reproductive season over four successive reproductive periods, until the deaths of all of them were recorded (February 1980). Since synchronization in the reproduction activity exists between branches (Rinkevich and Loya, 1979b), only one branch was sampled each time from each colony. This sampling procedure did not affect survivorship or reproduction (unpub.). Colony mortality was high (Table III), although colonies were carefully chosen on the basis of their healthy state (without dead branches or tissue damage). One and

TABLE III

Reproductive state and average number of female gonads per polyp in Stylophora pistillata colonies sampled during Dec. 1976 to Feb. 1980

Coral no.	Average numbers of female gonads per polyp in								
	Dec. 76	Apr. 77**	Dec. 77	Apr. 78	Jun. 78	Dec. 78	Apr. 79	Dec. 79	Feb. 80
1	-(10)	0.7 (10)*	D						
2	0.2 (10)	0.5 (10)*	D						
3	0.6 (10)	2.9 (10)*	1.2 (6)	2.6 (7)*	0.7 (6)*	-(10)	D		
4	2.4 (10)	1.4 (10)*	0.2 (6)	+(10)	D				
5	0.4 (10)	0.4 (10)*	D						
6	0.1 (10)	1.9 (10)*	+(6)	-(10)	-(10)	-(8)	D		
7	1.9 (10)	1.1 (10)*	0.5 (6)	D					
8	-(10)	D							
9	0.6 (10)	1.1 (10)*	0.7 (7)	2.2 (6)*	S				
10	-(10)	0.4 (10)*	+(6)	+(6)	-(8)	1.4 (7)	0.4 (7)	-(9)	D
11	-(10)	D							
12	-(10)	D							
13	-(10)	2.5 (10)*	2.1 (10)	2.5 (10)*	-(6)	1.8 (6)	1.0 (8)	D	
14	-(10)	0.5 (10)*	-(5)	1.9 (11)	-(10)	-(10)	-(10)	D	
15	-(10)	1.6 (10)*	1.0 (5)	S					
16	1.1 (10)	1.3 (10)*	2.3 (10)	S					
17	-(10)	D							
18	-(10)	0.4 (10)*	D						
19	-(10)	0.9 (10)*	-(11)	3.3 (7)	S				
20	0.2 (10)	0.9 (10)*	+(7)	2.1 (8)	-(10)	1.2 (6)	D		

December and February months refer to the beginning of the reproductive season, April months to the peak of reproduction, and June to the decline phase of the reproductive season (numbers in parentheses refer to the number of polyps examined).

-, Sterile colony; +, Only male gonads present; D, The colony was found dead; *, Planulae detected in histological sections; **, Planulae were found in all plankton nets put on marked colonies; S, Destroyed by storm.

two years after the beginning of the study, 60% and 30%, respectively, of the colonies were alive. Only one colony of the 20 samples (5%) remained alive after 3 years (Table III). A decrease in fecundity was repeatedly observed several months prior to the natural death of many of the colonies. In four out of the six dead colonies following a period of high fecundity (colonies 9, 15, 16, 19; Table III), the death was attributed to southern storms. None of the dead colonies during the first 4 months of the study (colonies 8, 11, 12, 17; Table III) contained any genital cells when first sampled.

The results (Table III) also indicate variability in sexuality (reproductive states: male, hermaphrodite, or sterile modes of reproduction) and fecundity of a specific colony in different years. Hermaphroditic colonies which exhibit high fecundity in one reproductive season may differ in the following reproductive season in which they become sterile (colonies 6, 14; Table III), or male (colonies 4, 10; Table III) and vice versa. The changes in colony reproductive patterns are further demonstrated in colonies sampled in three to four consecutive December months (colonies 3, 6, 10, 13, 14, 20; Table III). Sexuality or fecundity of five out of these six colonies were altered in each December month.

In the second set of experiments (Table IV), 155 large shallow water colonies were sampled over 10 successive sampling periods at the beginning and during the peak of 5 reproductive seasons (April 1976–April 1980). Changes among the different seasons

TABLE IV

Stylophora pistillata: reproductive states of shallow water populations

Date	No. of colonies	Colony reproductive state (%)			Average female gonads (polyp ⁻¹ colony ⁻¹)
		Hermaphrodites	Males only	Sterile	
Apr. 76	17	94	6	—	1.5 ± 0.9
Dec. 76	20	45	—	55	0.4 ± 0.7
Apr. 77	16	100	—	—	1.2 ± 0.8
Dec. 77	12	67	25	8	0.7 ± 0.8
Apr. 78	9	67	22	11	1.6 ± 1.3
Jun. 78	16	31	—	69	0.2 ± 0.4
Dec. 78	14	50	—	50	0.5 ± 0.6
Mar. 79	26	88	—	12	1.5 ± 1.1
Dec. 79	12	50	25	25	0.5 ± 0.7
Apr. 80	13	62	—	38	0.4 ± 0.6

either in sexuality or fecundity were observed. For example, each one of the 4 different December months (years 1976, 1977, 1978, 1979) represented different patterns of reproductive states: 8–56% of the colonies were sterile, 0–25% males and 45–67% were hermaphrodites among the different December months. The same pattern was recorded for sexuality of March to April months: 0–38%, 0–22% and 62–100%, respectively (Table IV). It is concluded that “one year of sampling” is not enough for the characterization of reproductive states in this species.

Reproduction in shallow versus deep water populations

Possible differences in reproduction between shallow and deep water populations were tested in two sets of experiments. In the first, we analyzed serial histological sections of 90 shallow water colonies (from Table IV). The results were compared to those of 77 deep water colonies (25–45 m, Table V) sampled on the same days during three successive reproductive seasons (April 1978–April 1980). Shallow water colonies possessed up to 5 times more female gonads per polyp per colony than deep water colonies ($P < 0.01$, Wilcoxon's signed rank test; Sokal and Rohlf, 1981). This phenomenon was most clear during the peak of the reproductive season, March to

TABLE V

Stylophora pistillata: reproductive states of deep water colonies

Date	Depth (m)	No. of colonies	Colony reproductive state (%)			Average female gonads (polyp ⁻¹ colony ⁻¹)
			Hermaphrodites	Males only	Sterile	
Mar. 78	60	1	100	—	—	0.7
Apr. 78	25–30	15	40	40	20	0.3 ± 0.4
Jun. 78	25	15	7	—	93	0.0 ± 0
Dec. 78	27–30	15	86	7	7	0.7 ± 0.6
Mar. 79	40–45	11	36	36	28	0.4 ± 0.8
Dec. 79	27–30	9	—	—	100	0
Apr. 80	25–30	12	8	50	42	0.0 ± 0.1

TABLE VI

Shed planulae in sample branches of shallow and deep water Stylophora pistillata colonies

Date	Shallow				Deep			
	Depth (m)	No. of colonies	Colonies which shed planulae (%)	Average no. of planulae (per 100 g skeleton)	Depth (m)	No. of colonies	Colonies which shed planulae (%)	Average no. of planulae (per 100 g skeleton)
Jan. 79	3-6	5	80	32 ± 49	27	6	0	0
Feb. 79	3-6	6	100	85 ± 95	27-30	4	25	1 ± 1
Mar. 79	5	6	100	22 ± 20	40-42	5	20	0.4 ± 1
Jun. 79	5	4	100	31 ± 22	34	5	20	2 ± 5
Dec. 79	5	4	0	0	30	3	0	0
Jan. 80	3-5	9	89	14 ± 31	39-42	9	33	4 ± 10
Feb. 80	5	6	83	4 ± 2	30	6	0	0
Apr. 80	3-6	10	90	30 ± 28	25-27	9	44	3 ± 6
May 80	3-8	10	90	44 ± 47	27	10	70	4 ± 7
Jun. 80	6-9	10	100	14 ± 10	27-30	10	30	3 ± 7
Jul. 80	4-6	10	70	3 ± 6	30	10	20	0.2 ± 0.4
Jan. 81	3-5	5	60	4 ± 8	30-35	5	0	0
Feb. 81	3-5	6	67	5 ± 3	27-30	6	33	1 ± 1

April (the average female gonad per polyp in April 1980 is lower than other April months because samples were taken immediately after a storm).

In the second set of experiments, planulae were collected in the laboratory from branch samples of 91 shallow water and 88 deep water colonies, on 13 collecting dates (Table VI). Significantly more planulae were shed by shallow water colonies than by deep water colonies ($P < 0.01$; Wilcoxon's signed rank test; Sokal and Rohlf, 1981). At the peak of the reproductive season about 20-80 planulae on average were shed per 100 g skeleton from shallow water colonies during one night. In deep water colonies the number did not exceed four planulae. An additional 55 deep water colonies were sampled during summer and fall (July-November) to examine whether the reproductive season there differs from that of shallow water populations. All histological sections were free of eggs. Moreover, no planulae were shed during a parallel study where branches were carefully broken *in situ* from an additional 80 colonies. These results indicate that the reproductive season of deep water colonies is probably two to three months shorter than that of shallow water populations.

DISCUSSION

The study of coral reproductive biology may be engaged with ambiguous definitions which could lead to wrong interpretations. For example, Fadlallah (1983) indicated that confusion arises from the applications of the term hermaphroditism, which describe two different life history processes: (1) development of monoecy over the lifetime of a specimen and, (2) sequential maturation of female and male products within one breeding period. Thus, it was accepted that *S. pistillata* (Rinkevich and Loya, 1979a, b) and *Goniastrea australensis* (Kojis and Quinn, 1981a, b) were protandrous hermaphrodites over their lifetime, but protogynous hermaphrodites in each single reproductive season. The present study indicates that either sexuality and/or fecundity may be completely altered from one reproductive season to the next. Her-

hermaphroditic colonies which exhibited high fecundity in one season became male or even sterile thereafter, and vice versa. Small colonies (geometric mean radius $\bar{r} < 2$ cm) which invest much energy in rapid growth (Loya, 1985), possess only male gonads in their first year of reproduction. An increase in colony size correlated with an increase in percentage of hermaphroditic colonies within the population (Rinkevich and Loya, 1979b). Reproduction of injured colonies of *S. pistillata* which invested energy in growth and regeneration was significantly reduced for at least two successive reproductive seasons after the fracturing event (Rinkevich, 1982). In addition, the fecundity of dying colonies was reduced several months before their death (Table III and Rinkevich and Loya, 1986), and dying colonies often changed their sexuality before their mortality and became male. Field experiments also demonstrated that the number of female gonads per polyp in *S. pistillata* was significantly reduced in colonies competing intraspecifically and the typical synchrony in reproduction among different branches of a given colony was changed and desynchronized (Rinkevich and Loya, 1985).

From the above results, we suggest that sexuality and fecundity in *S. pistillata* are responsive to the general state of health of the colony and its energetic limitations.

Studies also addressed reproduction/energy allocation questions in other coral reef species. Kojis and Quinn (1985) found lower fecundity in damaged *Goniastrea favulus* colonies compared to unharmed controls and suggested that this resulted from reallocation of resources to growth activities that would repair damaged tissue and cover the broken skeletons. Richmond (1984) indicated that reef corals may allocate energy into new tissue via budding for colony growth, or via planulation for production of new colonies. He found that colonies of *Pocillopora damicornis* at Enewetak atoll, Marshall Islands, allocated the majority of their reproductive energy into larva production while in the eastern Pacific the same species channels energy into colony growth. Thus, internal and/or external (see below) determinants may play a significant role in the expression of sexuality or fecundity in hermatypic corals, although the mechanisms are not yet understood.

Stimson (1978) proposed that coral species which release planulae are characteristic of shallow water environments such as reef flats, and hypothesized that shallow water species should planulate to facilitate early settlement in the parental habitat. Conversely, deep water corals should release eggs and sperm into the water to facilitate dispersal. More recent studies, however, suggest that the mode of reproduction is related to more complex factors than habitat alone (Harriott, 1983; Szmant, 1986). Thus it is of interest to study the mode of reproduction of the same species in two different depths. Karlson (1981) found a reduction in reproductive activity with increasing depth in two Jamaican species of *Zoanthus*. Kojis and Quinn (1983) further indicate that fecundity of *Acropora palifera* decreased with depth. Colonies at depths greater than 12 m had approximately half the fecundity of surface colonies. These studies support the results of the present study (Tables IV, V, VI) which indicate high differences in fecundity between shallow and deep water *S. pistillata* colonies.

The importance of available energy for reproduction is apparent from the decrease of fecundity in deep water populations. *S. pistillata* invests photosynthetically derived energy in reproduction (Rinkevich, 1982; Rinkevich and Loya, 1983). McCloskey and Muscatine (1984) found that the daily CZAR (the percentage contribution of zooxanthellae-translocated carbon to animal maintenance respiration) in *S. pistillata* in deep water was less than half of that in shallow water. Mean CZAR at 35 m was 78%, compared to 157% at 3 m. They also found that the decreased carbon availability to the host animal at 35 m was the consequence of both decreased net carbon fixation and decreased percentage of net fixed carbon translocated to the host.

Therefore, we suggest, that the generous daily carbon supply in shallow water colonies enables them to channel significantly more energy to reproduction than deep water colonies.

It is hard to separate the two explanations for the differences in reproductive activity between shallow and deep water colonies *e.g.*, energy limitations *versus* selective pressures. The present results point to energy limitation in deep water colonies rather than to the suggestion of selection pressures which favor high fecundity of shallow water colonies (Stimson, 1978).

This study provides for the first time results of long-term (1974–1984) experiments and observations on reproductive activities in a hermatypic coral. These and earlier (Rinkevich, 1982; Rinkevich and Loya, 1985; 1986) results indicate that sexuality as well as fecundity are determined and regulated by a variety of internal processes (such as the state of health of the colony, regeneration of broken branches, energy limitation, senescence, and death) and external, physical parameters (such as storm activities). The only other relevant papers on external parameters are the recent works of Jokiel (1985) and Jokiel *et al.* (1985) who suggested that environmental factors including temperature, salinity, tidal cycle, visible, and ultraviolet radiation influence the number of planulae released by *Pocillopora damicornis* per spawning cycle, as well as the synchronization of lunar release of planula larvae. Therefore it is concluded that reproductive activity in *S. pistillata* involves significant energetic expenditures that otherwise would be allocated into other physiological pathways such as growth and maintenance. As a result, any significant stress or diminution in energy resources affects at least one of the reproductive characteristics of this species. Physical and biological parameters have often been directly related to the distribution of a given species. However, the present study indicates that even reproduction can be directly altered as a result of the influence of these parameters. The changes in the reproductive activity of *S. pistillata* colonies in the field may not be a function of a single parameter. The combined effect of several external and internal factors on gravid colonies should be examined experimentally before and during the reproductive season. These should be considered before formulating any general hypothesis on coral reproduction.

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VITELLINS AND VITELLOGENINS OF THE TERRESTRIAL ISOPOD, *ARMADILLIDIUM VULGARE*

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ABSTRACT

Four forms of vitellogenin (Vg·1–Vg·4) in the hemolymph and four forms of vitellin (Vn·1–Vn·4) in the oocytes of reproductive females of *Armadillidium vulgare* were detected by polyacrylamide gel electrophoresis (PAGE) at stage D of the molting cycle. All vitellogenins decreased and were not detected in the hemolymph at stage E. At stage E, Vn·1–Vn·3 disappeared; Vn·4 was the major vitellin component in mature oocytes. The electrophoretic patterns of vitellogenin and vitellin revealed changes correlated with the molting cycle and oocyte growth.

Vitellogenins were electrophoretically identical to vitellins. Rabbit anti-Vn·4 antibody cross-reacted with vitellogenins. Using Slater's method, the four forms of vitellin were glycolipoproteins, with molecular weights of Vn·1–700,000, Vn·2–620,000, Vn·3–540,000, and Vn·4–470,000. In SDS-PAGE, vitellin Vn·4 yielded three main polypeptide components with molecular weights of 80,000, 99,000, and 127,000. The polypeptide compositions among vitellins (Vn·1–Vn·4) were similar.

INTRODUCTION

The correlation between the molting cycle and secondary vitellogenesis was described in the amphipod *Orchestia gammarellus* (Meusy and Charniaux-Cotton, 1984). In the isopod *Porcellio dilatatus*, the fat body (subepidermal adipose tissue) is the site of vitellogenin synthesis (Picaud and Souty, 1980). The cauterization of Y-organs (Souty *et al.*, 1982; Picaud, 1983) and ovariectomy (Picaud and Souty, 1981) of *P. dilatatus* lowered the rate of vitellogenin release into the hemolymph. However, no study elucidates the hormonal mechanisms which regulate the vitellogenin synthesis in Crustacea.

I am investigating the hormonal control of reproduction—especially hormonal regulation of vitellogenin synthesis—in *Armadillidium vulgare*. Previous work on *A. vulgare* indicated that rapid oocyte growth occurs at stage D of the molting cycle. Oocytes did not continue to grow in Y-organ ablated females (Suzuki, 1986). Y-organ (molting hormone) was needed for oocyte growth. However, it is not yet known whether vitellogenin synthesis is induced by molting hormone in *A. vulgare*.

The present study identified female-specific proteins (vitellins and vitellogenins) of *A. vulgare* by electrophoresis. The data will contribute to the understanding of vitellogenin synthesis and its hormonal (molting hormone) regulation. This paper identifies four forms of vitellogenins and vitellins, and describes their characteristics in *A. vulgare*. Three of the forms were described by Picaud (1983).

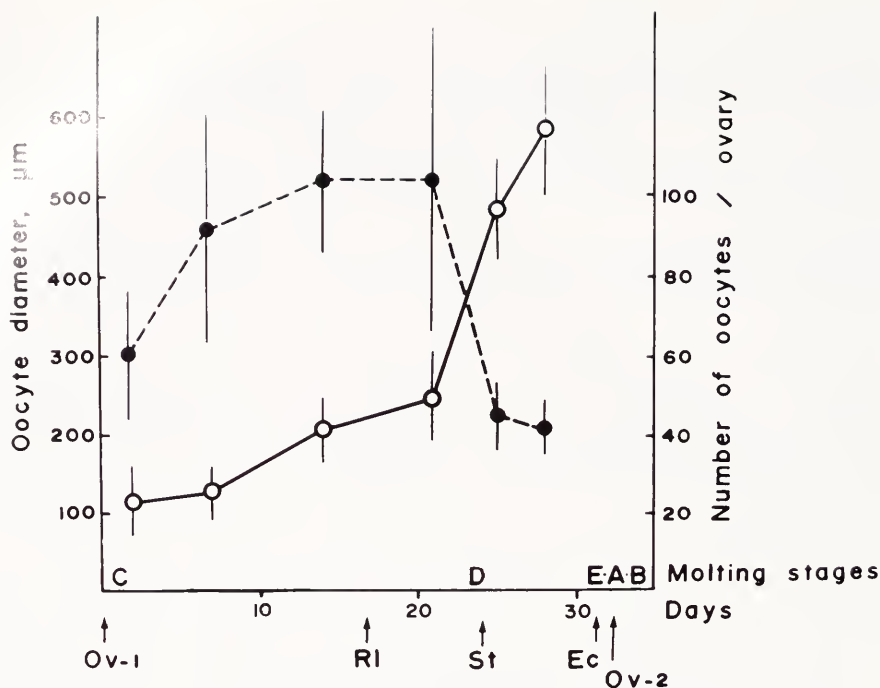


FIGURE 1. Oocyte growth during the molting cycle following first oviposition in *Armadillidium vulgare*. —○—, diameter of oocyte; --●--, number of oocytes; Ov-1, first oviposition; Ov-2, second oviposition; RI, release of larva; St, sternolite; Ec, ecdysis. Vertical lines around the points show standard errors.

MATERIALS AND METHODS

Animals

Armadillidium vulgare was collected near Yokohama in early March 1986. Several females (10–12 mm body length) were kept in a petri dish with moistened soil. Two adult males were added to the dish for a normal reproductive cycle. They were maintained at $25 \pm 2^\circ\text{C}$ in natural daylight, and fed decayed leaves and rat chows.

To observe oocyte growth, fresh ovaries were examined microscopically.

Preparation of hemolymph and ovarian homogenate to electrophoresis

Hemolymph samples were collected using a capillary tube placed in a hole in the tergite of the seventh thoracic segment. It was diluted 1/10 with Tris-HCl buffer (20 mM, pH 7.6 containing 130 mM NaCl and 5 mM EDTA). Dissected ovaries were rinsed and homogenated in 300 μl Tris-HCl buffer. Ovarian homogenate was passed through prefilter (mipore, AP) to remove lipids, centrifuged at $20,000 \times g$ for 15 min at 4°C , and the supernatant was collected as a sample of ovarian homogenate.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using 5% acrylamide monomer (Davis, 1964). Polyacrylamide gel electrophoresis in sodium dodecyl sul-

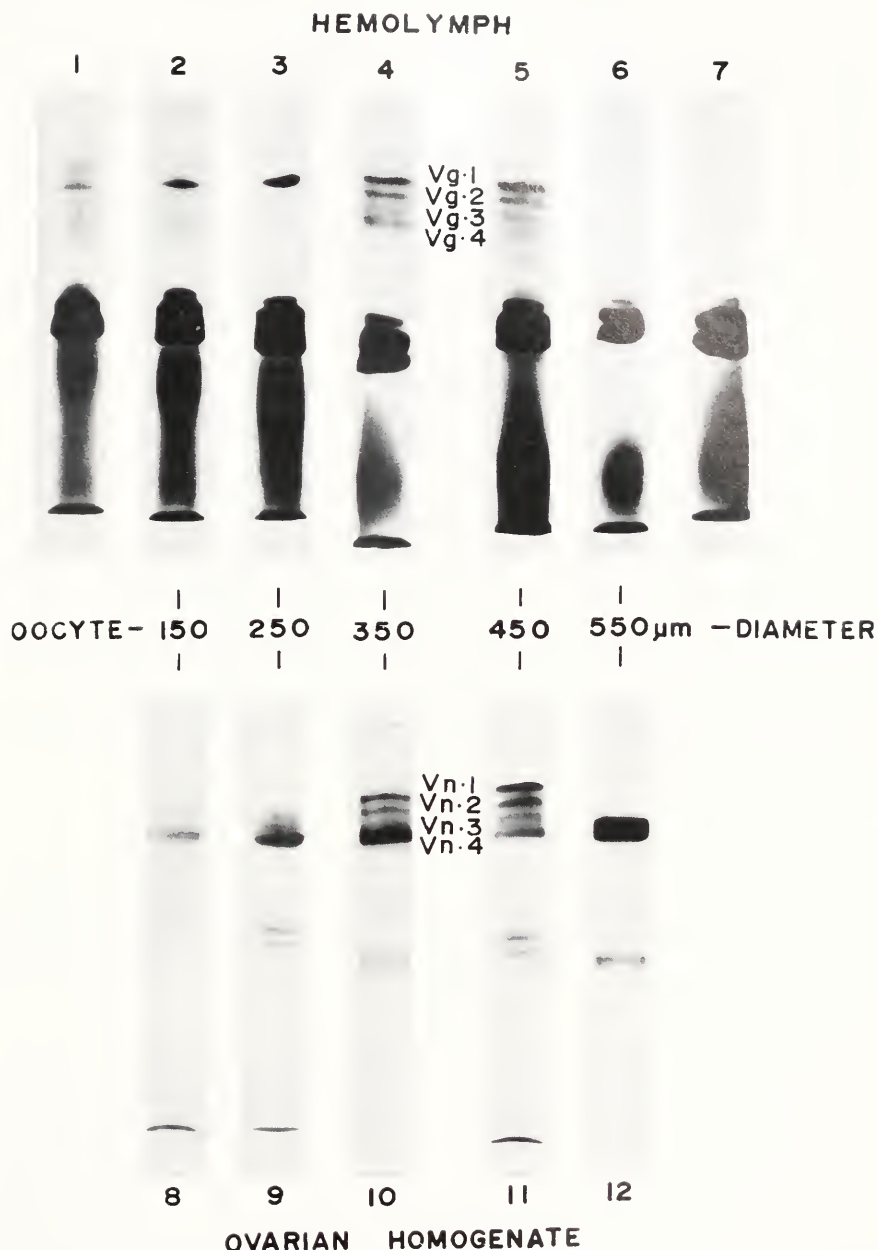


FIGURE 2. Electrophoretic pattern of the hemolymph and the ovarian homogenate on 5% PAGE. 1, hibernating adult females; 2-6 and 8-12, vitellogenic females; 7, adult male; Vg-1-Vg-4, vitellogenin; Vn-1-Vn-4, vitellin.

fate (SDS-PAGE) was also performed (Laemmli, 1970) using 10% acrylamide. Pore-limited electrophoresis was carried out on 5-25% acrylamide gradient slab gels (PAGGE) (Slater, 1969) using Davis's buffer system.



FIGURE 3. PAGE of the ovarian homogenate (350–450 μm in oocyte diameter) stained for protein with Coomassie blue (1), for glycoprotein with periodic acid/Schiff reagent (2), and for lipoprotein with Sudan black B (3).

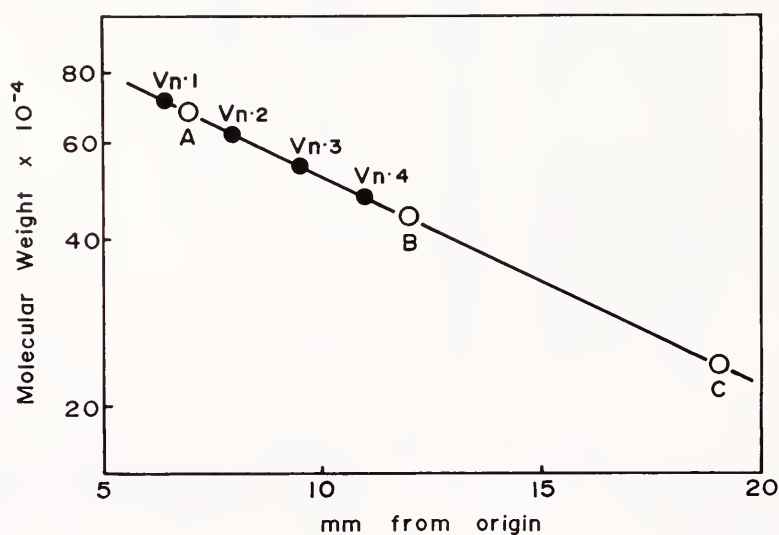


FIGURE 4. Molecular weight determination of vitellins by pore-limited electrophoresis (5–25% PAGGE) of ovarian homogenate (350–450 μm). Molecular weights were calculated from mobilities relative to each standard proteins (A–C, Pharmacia). A, thyroglobulin (669,000 M. W.); B, ferritin (440,000); C, catalase (232,000); Vn.1–700,000; Vn.2–620,000; Vn.3–540,000; Vn.4–470,000.

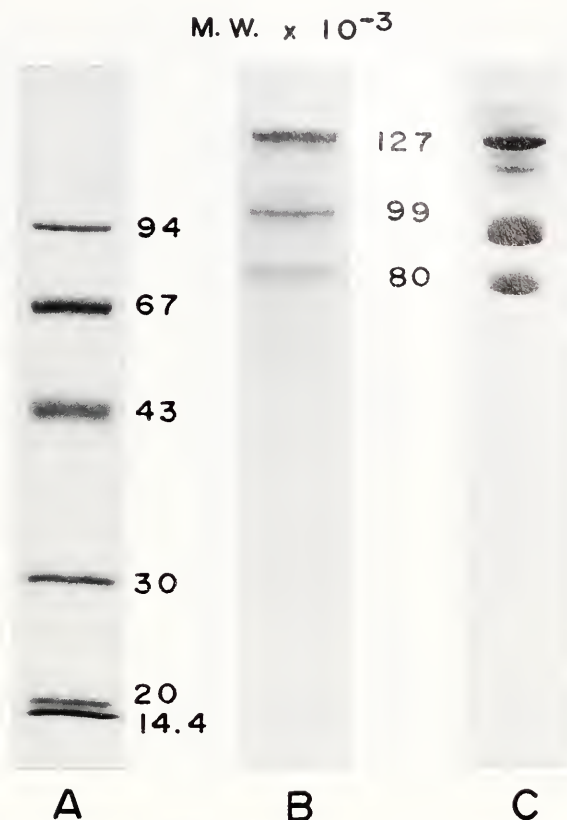


FIGURE 5. Molecular weight determination of Vn-4 polypeptide by SDS-PAGE of ovarian homogenate. A, slab gel of standard proteins (phosphorylase b-94,000; bovine serum albumine-67,000; ovalbumin-43,000; carbonic anhydrase-30,000; soybean trypsin inhibitor-20,000; α -lactalbumin-14,400, Pharmacia). B, slab gel of ovarian homogenate (150 μm in oocyte diameter and stage C). C, disc gel of ovarian homogenate (500 μm and stage E).

After electrophoresis, proteins were stained with Coomassie blue and glycoproteins were visualized using the periodic acid Schiff (PAS) method (Zacharius *et al.*, 1969). Lipoprotein samples were stained with Sudan black B (Sano, 1981) prior to electrophoresis.

Preparation of antibody and immunodiffusion test

VN-4 was used as antigen. Mature oocytes (200, 550 μm in diameter) were homogenized in Tris-HCl buffer and centrifuged. The resulting 1.5 ml containing about 1 mg/ml total proteins (Lowry *et al.*, 1951, with BSA as a standard) was applied to the top of a slab gel (PAGE, 3 mm thick, 10.5 $\times$ 11.5 cm, 30 mA for 2 h). After electrophoresis, both sides of the gel were stained with Coomassie blue to define the vitellin band. The Vn-4 band was then cut from the unstained portion of the slab gel and thoroughly homogenized with 1.5 ml Tris-HCl buffer. The gel homogenate (3 ml) was stored frozen at $-80^{\circ}C$ until use.

One milliliter of the homogenate (350 μg /ml proteins) and Freund's complete

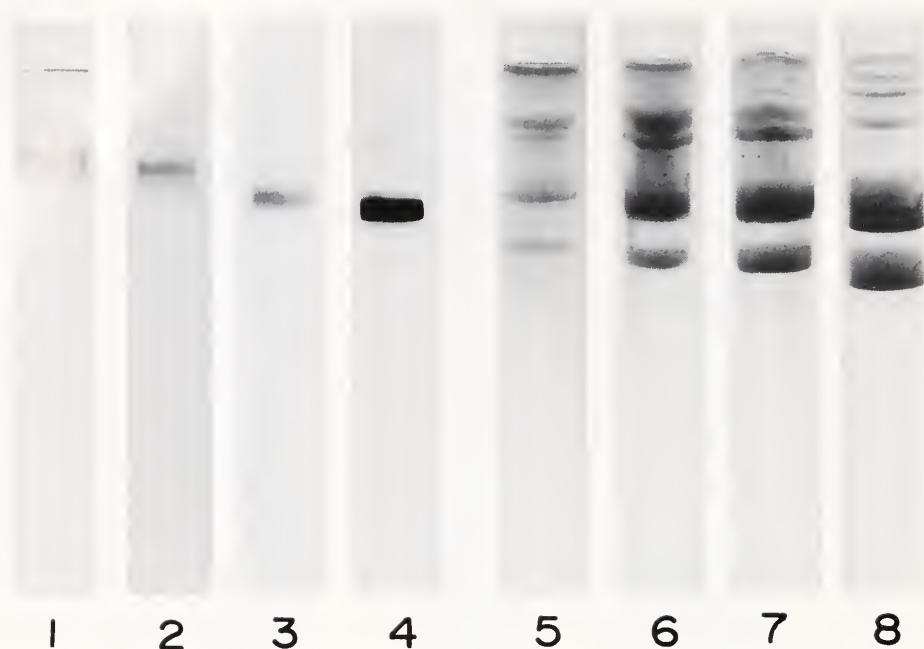


FIGURE 6. Polypeptide analysis of vitellin Vn-1-Vn-4. Ovarian homogenate (350–450 μ m) was prestained with Sudan black B. After PAGE of homogenate, each vitellin band was cut out of the disc gels and eluted by homogenizing the gel bands in 20 mM Tris-HCl buffer. PAGE patterns of separated Vn-1 (1), Vn-2 (2), Vn-3 (3), and Vn-4 (4). Polypeptide analysis of each separated Vn-1 (5), Vn-2 (6), Vn-3 (7), and Vn-4 (8) on disc SDS-PAGE.

adjuvant (1 ml) were injected subcutaneously into the back of a male rabbit twice every 10 days. A booster injection was given once, on the 10th day after the second antigen injection. Five days later the rabbit was bled and an immunoglobulin (IgG) fraction was precipitated by ammonium sulfate (40% saturation). After dialysis against Tris-HCl buffer, this antiserum (anti-Vn-4 IgG) was stored frozen at -80°C .

Immunodiffusion tests were performed (Ouchterlony, 1949). Samples were tested on 1% agarose gel plates with 0.5 M Tris-HCl buffer. Both antigen and antibody were allowed to diffuse for one day at room temperature and examined for precipitin lines.

RESULTS

Changes of vitellin and vitellogenin during the molting cycle

Young oocytes were seen (Fig. 1) in the ovaries after oviposition. They gradually increased in size during stage C of the molting cycle. Rapid oocyte growth began soon after the appearance of the sternoliths, which were evident in stage D. Oocytes were about 550 μ m at stage E, and ecdysis occurred in the posterior and then anterior region of the female's body before oviposition.

The electrophoretic pattern of vitellogenins and vitellins was investigated on disc PAGE. Diluted hemolymph (20 μ l) and ovarian homogenate (30 μ l) were subjected to electrophoresis at 2 mA per tube for 3 h. Hemolymph analysis revealed four forms of vitellogenin (Vg-1-Vg-4) present in the hemolymph of stage D females with sub-

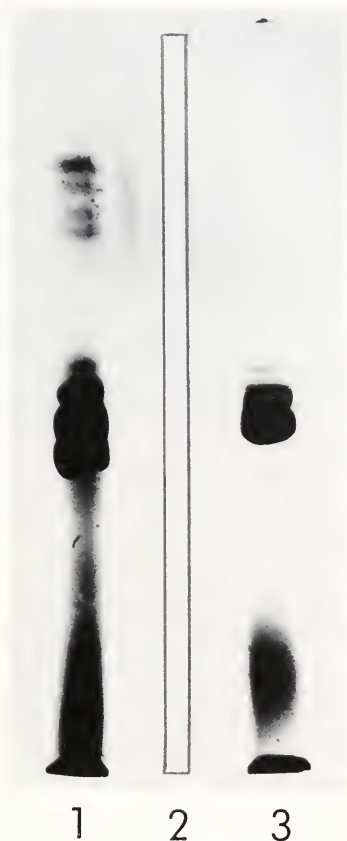


FIGURE 7. Identification of vitellogenin by immunoelectrophoresis. Hemolymph from reproductive female (1) and male (3) at stage D of the molting cycle were subjected to PAGE. Disc gels were then embedded in a layer of 1% agarose gel on a glass plate. The trough (2) of the agarose gel was cut and filled with anti-Vn·4 serum. After 24 h diffusion at room temperature, PAGE gels of (1 and 3) were removed and the precipitate line of immunochemical reaction was stained with Coomassie blue. The other PAGE gels of (1 and 3), stained with Coomassie blue, were placed on each original site of the agarose gel.

mature oocytes (350–450 μm) (Fig. 2·4, 5). These forms were not detected in females with mature oocytes (550 μm) at stage E of the molting cycle (Fig. 2·6). Vitellogenin was not detected either in stages A and B of the reproductive females or in the hemolymph of males (Fig. 2·7) throughout the molting cycle.

Analysis of ovarian homogenate revealed four forms of vitellin (Vn·1–Vn·4) in immature oocytes of stage D females (Fig. 2·10, 11). These forms of vitellin exhibited the same electrophoretic pattern as vitellogenin. At stage E, Vn·1–Vn·3 were not detected; Vn·4 was the major component of vitellin found in mature oocytes (Fig. 2·12). Vn·4 was also the primary vitellin in young oocytes of stage C females (Fig. 2·8, 9).

Characterization of vitellogenins and vitellins

The four bands of vitellin and vitellogenin had low electrophoretic mobility and were located closely together (Fig. 2·5, 11). Figure 3 shows that the vitellins were



FIGURE 8. Ouchterlony agar diffusion analysis showing reaction between anti-Vn.4 serum (A) against ovarian homogenate (1 and 2) or hemolymph of females (3-6). 1 and 4, stage C; 2 and 5, stage D; 3, hibernating; 6, stage E.

glycolipoproteins, as stained with periodic acid/Schiff reagent and Sudan black B. The ovarian homogenate, prepared from immature oocytes (350–450 μm), was analyzed by the pore-limited method (PAGE) using standard proteins to determine the molecular weight of the four vitellins (Fig. 4). They were estimated to be Vn.1–700,000 (700K), Vn.2–620,000 (620K), Vn.3–540,000 (540K), and Vn.4–470,000 (470K), respectively.

Figures 5 and 6 show the polypeptide composition of vitellin separated in SDS-PAGE. Vn.4, the major vitellin in stages C and E (Fig. 2·8, 12), yielded three main polypeptides with molecular weights estimated to be 80,000 (80K), 99,000 (99K), and 127,000 (127K) (Fig. 5). To compare the polypeptide compositions of Vn.1–Vn.4, each vitellin prestained with Sudan black B was isolated from immature oocytes using PAGE. After electrophoresis, the gel band of each vitellin was cut out and eluted in Tris-HCl buffer. Although the isolated vitellin overlapped with other vitellins (Fig. 6·1–4) each vitellin was analyzed on SDS-PAGE. Vn.1–Vn.4 were separated into several polypeptides; their main polypeptide compositions were very similar (Fig. 6·5–8). The lower polypeptides (MW 80K and 99K) were common through Vn.1–Vn.4 (Figs. 5, 6).

Immunochemical reactions of anti-Vn.4 serum against ovarian homogenate (vitellin) and diluted hemolymph (vitellogenin) were examined by double diffusion analysis in agarose gel. The four vitellogenins gave a single precipitate line when anti-Vn.4 serum was reacted with the hemolymph of stage D females. The precipitate line was absent when the serum was reacted with hemolymph of males (Fig. 7). Figure 8 presents the reactions between anti-Vn.4 serum and various stage hemolymph or ovarian homogenates. These results indicate that anti-Vn.4 antibody cross-reacted with vitellins and vitellogenins.

DISCUSSION

Four forms of vitellin and vitellogenin were identified electrophoretically from *Armadillidium vulgare*. Picaud (1983) found three forms from this species by electrophoresis. Vitellins are glycolipoproteins with higher molecular weights (700K–470K).

Vn·4, the smallest vitellin, contains three main polypeptides at MW 80K, 99K, and 127K. The polypeptide compositions of Vn·1–Vn·4 are quite similar. Vitellins of the isopods *Porcellio dilatatus* (315K), *Idotea balthica* (290K), and *Ligia oceanica* (320K) have similar molecular weights (Picaud, 1983); molecular weights of *A. vulgare* vitellins are higher. However, the main polypeptide compositions of Vn·4 from *A. vulgare* (80K, 99K, and 127K) have molecular weights similar to those of *P. dilatatus* (78K, 97K, and 180K) (Picaud, 1983).

The presence of a precursor (vitellogenin) of egg yolk protein (vitellin) shows that *A. vulgare* vitellin appears to be synthesized at an extra ovarian site (possibly the fat body) and then transported to the ovary through the hemolymph, as in *Porcellio dilatatus* (Picaud and Souty, 1980). However, Souty (1983) reported that the ovary of *P. dilatatus* can synthesize its proteinic yolk.

Changes of vitellin and vitellogenin were observed during the molting cycle and oocyte growth. All forms of vitellin and vitellogenin were observed at stage D and, except for Vn·4, they declined and disappeared during oocyte growth. The major peak of vitellogenin synthesis was observed at stage D of the isopod in *Idotea balthica* by Souty and Picaud (1981) and in *Porcellio dilatatus* by Picaud and Souty (1981). The question remains whether there is a correlation between the changes in the appearance of vitellin and/or vitellogenin and the Y-organ (molting hormone) in *A. vulgare*. In isopods, however, Charniaux-Cotton (1985) found that molting hormone is necessary for vitellogenin synthesis and is regulated by a feedback mechanism.

Since vitellin Vn·1–Vn·4 have common polypeptides and a common antigenic determinant, the smallest vitellin (Vn·4) may be accumulated throughout oocyte growth. Vn·1–Vn·3 seem to undergo a proteolytic processing in the oocytes. Analogous phenomena have been reported (Baert, 1985, 1986) for *Pereneris cultrifera* (polychaete). During oocyte growth, the higher molecular weight vitellins disappeared leaving the single form (the lowest vitellin) at immaturity. These results suggest that a progressive proteolytic cleavage of vitellin occurs in polychaetes. Vitellogenin processing has also been reported in amphipods (Junéra and Meusy, 1982) and in the locust (Chen *et al.*, 1978). The processing of vitellin and vitellogenin is presently unclear in *A. vulgare* and awaits further study.

ACKNOWLEDGMENTS

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A SCANNING ELECTRON MICROSCOPE STUDY OF *ASCIDIA MALACA* EGG (TUNICATE). CHANGES IN THE CELL SURFACE MORPHOLOGY AT FERTILIZATION

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ABSTRACT

Ascidia malaca eggs with and without envelopes were studied using the scanning electron microscope. Follicle cells, chorion, and test cells were examined and compared with those of other ascidian species. No appreciable differences were found. The surface topography of dechorionated eggs differed before and after fertilization.

The pole of the unfertilized egg was indicated by a small smooth region beneath the polar pit. The remaining surface of the egg was undulated. Short microvilli were scattered on the cell membrane except in the area nearest the polar pit. Surface displacements occurred in the fertilized egg, changing its morphological features, at various intervals after sperm penetration. The smooth region expanded shortly after fertilization, and the inferior part of the vegetal hemisphere was corrugated by pronounced undulations. Two new types of microvilli appeared. After the ejection of the first polar body the appearance of the egg surface changed: the animal hemisphere became corrugated, bearing numerous short microvilli, and the vegetal hemisphere showed slight undulations. At the vegetal pole microvilli concentrated to form a protuberance. After the ejection of the second polar body a diffusion of elongated microvilli was observed. The present results indicate that during ooplasmic segregation the movement of the cell membrane components produces changes in the surface topography. These govern the rearrangement of the cytoplasm.

INTRODUCTION

Ascidian eggs show characteristic deformations at fertilization and in the interval between the ejection of the first and second polar bodies. Cell surface movements after sperm penetration were studied by Ortolani (1955). Colored chalk granules were bound to the plasma membrane to mark the surface of the unfertilized egg. After fertilization their displacement was observed using the light microscope; the polar pit was used as a landmark. Her observations suggested that the movements of the surface and the modifications in shape were caused by a cortical contraction.

The present SEM study examined the morphology of *Ascidia malaca* eggs with and without envelopes. The dechorionated egg was examined both before and after fertilization to observe the modifications which characterize the surface topography. We followed the distribution and organization of the microvilli at different intervals after sperm penetration.

Previous ultrastructural investigations of *Ascidia malaca* eggs have been performed, but they were primarily concerned with oogenesis (Materazzi and Bondi, 1973; Gianguzza and Dolcemascolo, 1978; 1979), egg morphology (La Spina

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Abbreviations: SEM = Scanning electron microscopy; TEM = Transmission electron microscopy.

D'Anna, 1974), sperm morphology (Villa, 1975; Villa and Tripepi, 1983), and egg-sperm interaction (Villa, 1977). No information exists on the egg plasma membrane.

Transmission and scanning electron microscope studies of egg surface changes and early events in ascidian development used only *Ciona intestinalis* (Sawada and Osanai, 1981). The primary objective of this study was to elucidate the mechanism of ooplasmic segregation. The present results not only extend our knowledge of the ascidian egg envelope but also reveal differences in the egg surface between the animal and vegetal hemispheres, which become more evident after fertilization. During ooplasmic segregation significant changes and apparent transposition of the features of the two halves take place. Modifications in the distribution and shape of the surface microvilli continue to occur until the ejection of the second polar body.

MATERIALS AND METHODS

Adult specimens of *Ascidia malaca* were collected from the Gulf of Palermo. Male and female gametes were obtained surgically from gonoducts of dissected animals. Eggs removed from the oviducts were washed in Millipore-filtered seawater; some were left intact with egg envelopes, others were transferred to agar-coated Syracuse dishes and dechorionated by hand using steel needles.

The experiments were performed at 22°C by fixing unfertilized eggs and eggs 3, 7, and 30 minutes after insemination.

Transmission electron microscopy

For conventional TEM studies, unfertilized and fertilized (3 min after insemination) intact eggs were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer in seawater (pH 7.2) containing 4% sucrose for 30 min at room temperature, and postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C. The specimens were dehydrated in an ethanol-propylene oxide series and embedded in Dow epoxy resins (Lockwood, 1964). Sections were stained with saturated uranyl acetate and lead citrate (Venable and Coggeshall, 1965), and examined with a Siemens Elmiskop 1b TEM operating at 80 kV.

Scanning electron microscopy

Unfertilized and fertilized intact and hand-dechorionated eggs were fixed as described for TEM, then ethanol-dehydrated, critical-point dried, sputter-coated with gold, and observed with JEOL JSM 15 and ISI DS 130 SEMs. Other intact eggs were fixed and critical-point dried as above, but before they were coated with gold the follicle cell layer and chorion were partially dissected with a fine sharp needle. The dissected specimens were coated and viewed under the SEM as described above.

Intact and dechorionated eggs show no evidence of distortion after specimen preparation; however shrinkage is generally observed (about 20%). Shrinkage is greater in intact eggs since the chorion is greatly reduced by SEM preparation.

RESULTS

Egg envelope morphology

The *Ascidia malaca* egg envelope consists of an acellular layer—the vitelline coat (i.e. chorion)—lying between two cellular layers, the external follicle cells and internal test cells (Figs. 1–3, 5, 6). The follicle cells constitute a single layer of conical-

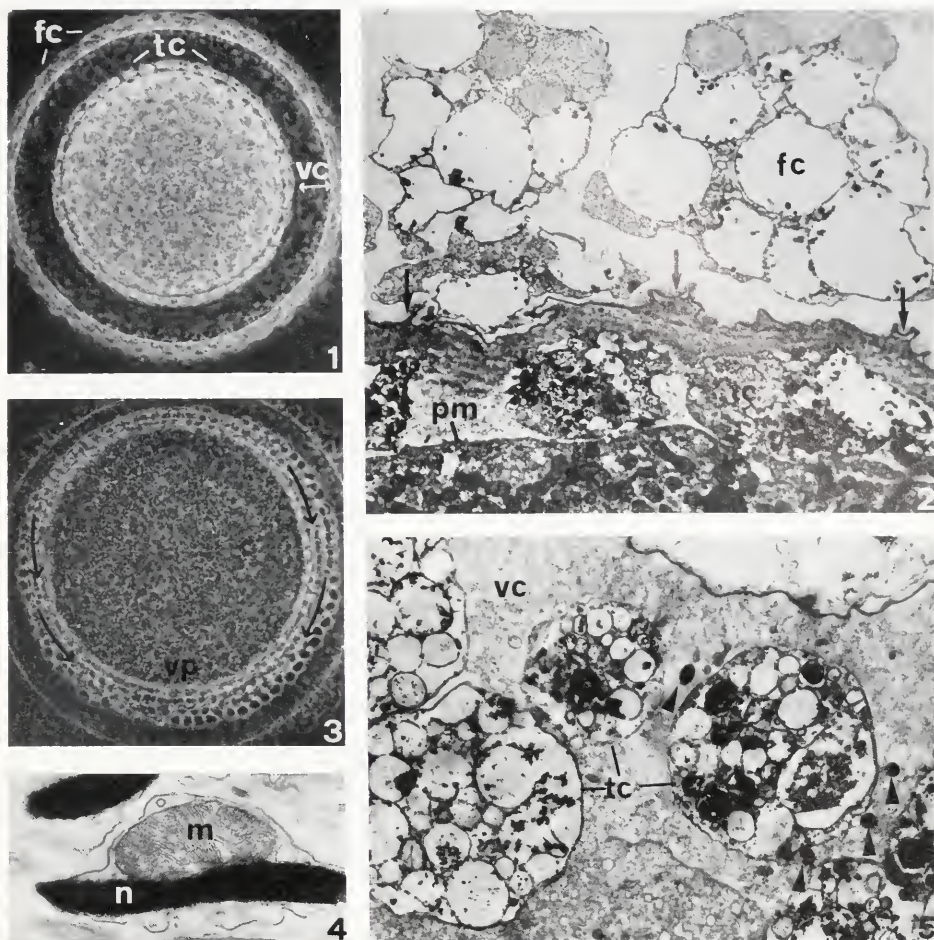


FIGURE 1. Phase contrast micrograph of a living unfertilized *Ascidia malaca* egg. fc = follicle cell; tc = test cell; vc = vitelline coat. 270 $\times$.

FIGURE 2. Transmission electron micrograph (TEM) of envelopes of unfertilized egg. Arrows indicate coating of the outer vitelline coat; pm = plasma membrane. 2500 $\times$.

FIGURE 3. Phase contrast micrograph of a living fertilized egg; test cells have migrated towards the vegetal hemisphere (arrows). vp = vegetal pole. 290 $\times$.

FIGURE 4. TEM of a head of a fully differentiated spermatozoon from the sperm duct. m = mitochondrion; n = nucleus. 10,000 $\times$.

FIGURE 5. TEM of envelopes of a fertilized egg; spermatozoa are found in the vitelline coat (arrow-heads). 5000 $\times$.

shaped, highly vacuolated hexagonally arranged cells; their convex basal region forms indentations in the vitelline coat, which are clearly seen when the latter is cut away (Fig. 11). The follicle cells touch one another only at their basal region, where the plasma membranes of adjacent cells seem to interdigitate, separated by narrow clefts.

The clefts are the only means by which the sperm can reach the subjacent vitelline coat, where the "sperm reaction"—involving swelling, migration, and loss of the mitochondrion—occurs. In fact, many "remnants" of spermatozoa (*i.e.* mitochondrion plus tail), and only a few intact ones, are found mainly in the cleft zone 3 minutes

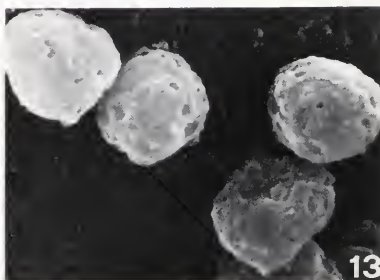
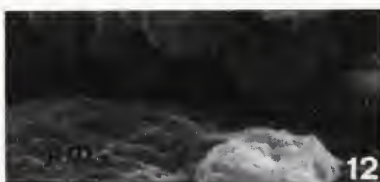
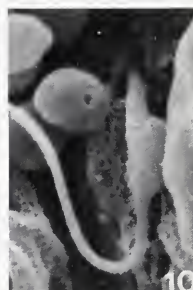
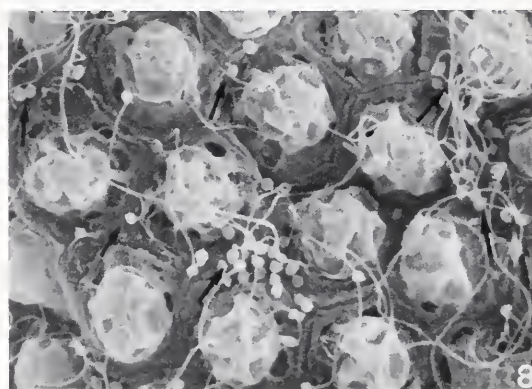
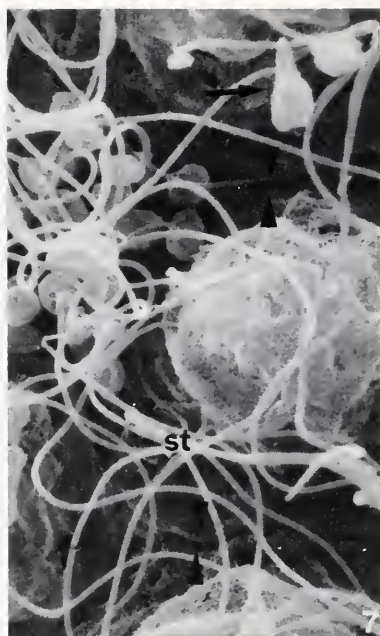


FIGURE 6. Scanning electron micrograph (SEM) of a fertilized egg; follicle cells form a hexagonal pattern (asterisks). 520X.

after fertilization (Figs. 7–10). At high magnification the follicle cells show a reticulated lace-like membrane (Fig. 7).

The chorion shows different morphological features and different thicknesses depending on fixation techniques; however, a thin homogeneous electron-dense outer layer with an organized coating (previously called "chorial membrane," Villa, 1977) and a thicker fibrous inner layer are always observed (Figs. 2, 5). The thickness of the chorion (*ca.* 20 μm *in vivo*) decreases during TEM preparation, and even further during that for SEM.

The chorion encloses the test cells that, in unfertilized eggs, are close to the egg plasma membrane. The test cells form a single but discontinuous layer of roundish or oval, moderately vacuolated cells which move freely within the texture of the chorion.

In fertilized eggs the test cells migrate towards the vegetal hemisphere where they accumulate in layers (Figs. 3, 5).

SEM examination of eggs in which the follicle cell and chorion layers had been partially cut away prior to gold coating revealed that the test cell plasma membranes have either pseudopodia-like extensions or invaginations of corresponding size (Fig. 11); holes can also be observed in the plasma membrane where the numerous microvilli would normally be located (Figs. 12, 13).

Surface morphology

Unfertilized egg. A well-defined polarity is observed: the plasma membrane at the tip of the animal hemisphere is slightly undulated, while the remaining parts are highly undulated with surface folds of random orientation (Fig. 14). The animal pole is marked by a pit, from the bottom of which the first polar body will emerge (Fig. 15). Short microvilli (approximately 0.2 μm in length) are fairly uniformly distributed on the egg surface (Fig. 16) except in the region of the polar pit that is relatively devoid of microvilli.

Eggs 3 min after insemination. The shape of the fertilized egg changes rapidly (Fig. 17): it elongates, becoming pear-shaped with transient bulges, and then rapidly regains its original shape. A marked polarity of the folded areas and two new types of microvilli also characterize this stage. The animal hemisphere and the upper half of the vegetal hemisphere are slightly undulated, while the lower half of the vegetal hemisphere is corrugated (Fig. 19); short microvilli, similar to those of the unfertilized eggs, are scattered over the entire surface except in the polar pit. Moreover, a few

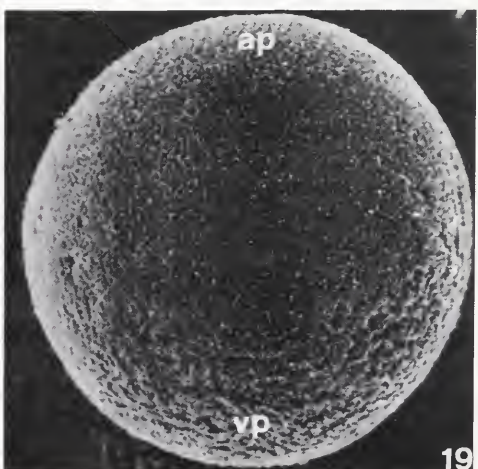
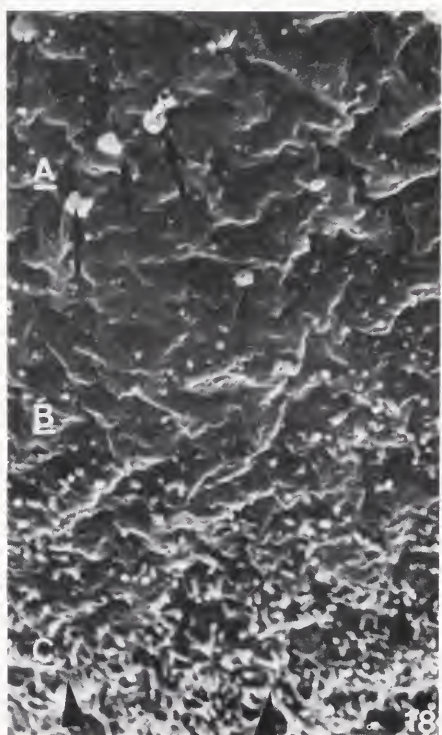
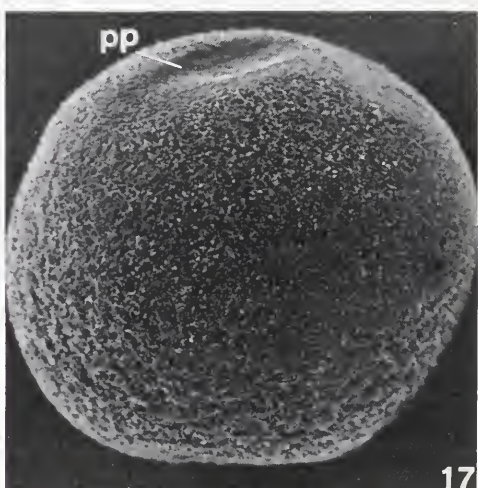
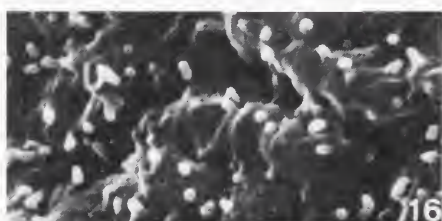
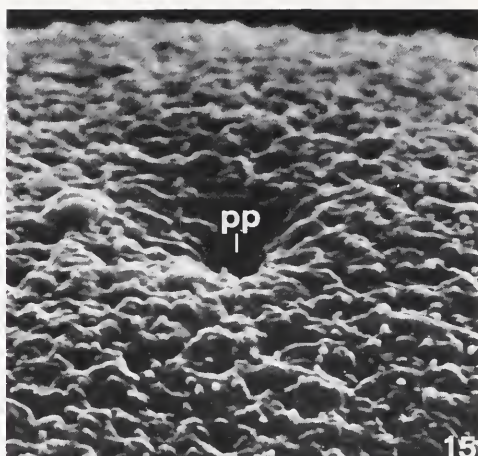
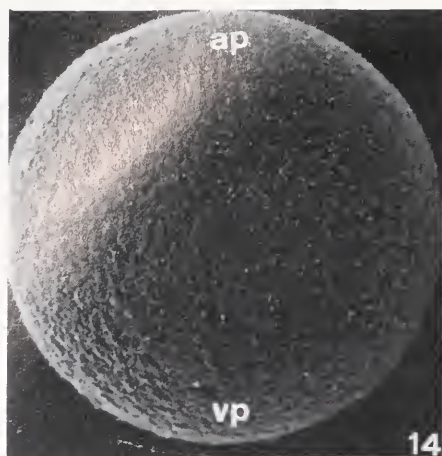
FIGURE 7. High magnification of the follicle cell layer showing clefts between the cells and lace-like reticular membrane. Arrow indicates an intact spermatozoon; arrowheads indicate clefts. st = sperm tail. 3000 $\times$.

FIGURE 8. SEM of the follicle cell layer of a fertilized egg. Arrows indicate numerous remnants of spermatozoa (*i.e.*, tail + mitochondrion) in the clefts between adjacent follicle cells. 1000 $\times$.

FIGURE 9. SEM of the cleft zone showing intact sperm (arrowhead) and two spermatozoa beginning the "sperm reaction" (arrows). 2000 $\times$.

FIGURE 10. High magnification of the cleft zone showing mitochondria left out and a still intact spermatozoon. 4500 $\times$.

FIGURES 11–13. SEM of unfertilized eggs in which follicle cells and vitelline coat were partially cut away. Figure 11: View of the underside of the vitelline coat showing indentations of the follicle cells (asterisks); numerous test cells with pseudopodia-like extensions or with invaginations (arrowheads) are left on the vitelline coat during dissection. 2710 $\times$. Figures 12, 13: View of the egg plasma membrane, on which some test cells rest, showing numerous pore-like openings corresponding to the base of the microvilli. Figure 12, 2050 $\times$. Figure 13, 3150 $\times$.



FIGURES 14–16. SEM of unfertilized eggs showing polarity (Fig. 14), smooth and slightly microvillated surface of the egg near the polar pit (Fig. 15), and high magnification of microvilli of highly undulated vegetal area (Fig. 16). ap = animal pole; pp = polar pit. Figure 14, 700 $\times$. Figure 15, 5460 $\times$. Figure 16, 7600 $\times$.

large stumpy microvilli (approximately $0.8\ \mu\text{m}$ in length) are randomly scattered in the equatorial area, and numerous slender microvilli ($0.5\ \mu\text{m}$ by $0.1\ \mu\text{m}$) can be observed very densely arranged in the lower half of the vegetal hemisphere (Fig. 18).

Eggs 7 min after insemination. After the ejection of the first polar body, striking but transient modifications in shape—such as a lobe formation at the vegetal pole (Fig. 21)—occur. An extensive reorganization of the egg surface results in the rearrangement of microvilli and in the apparent transposition of the folded areas from the vegetal to the animal hemisphere. The plasma membrane is highly undulated by pronounced folds in the animal hemisphere (Fig. 20), while in the vegetal hemisphere it is only slightly wrinkled (Fig. 22). The stumpy microvilli of the previous stage are still found in the equatorial area (Fig. 23); the slender microvilli also persist in the vegetal half. At the vegetal pole a well-defined circular protuberance covered by a dense clump of short microvilli appears (Figs. 22, 24); spermatozoa are occasionally encountered on the pole or in the surrounding region.

Eggs 30 min after insemination. The egg regains its spherical shape after ejection of the second polar body. The distribution of the folded areas is similar to that of the previous stage (Fig. 25), although the microvilli reorganize; they are again uniformly distributed over the whole surface and appear slightly longer when compared with those of the previous stages (Fig. 27). Moreover the numerous spermatozoa left on the plasma membrane after insemination are now concentrated at the vegetal pole in which the protuberance of clumped microvilli has gradually faded (Figs. 26, 28).

DISCUSSION

This study provides further information on the morphology of the accessory cells, and new data on nude surface topography.

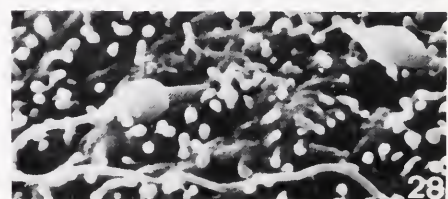
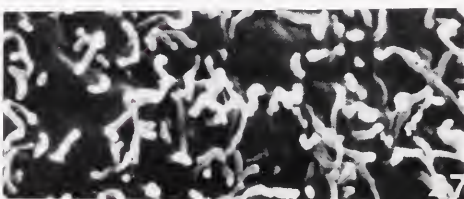
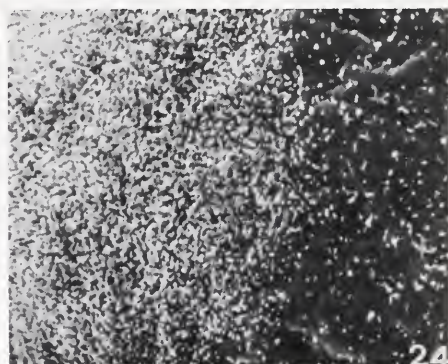
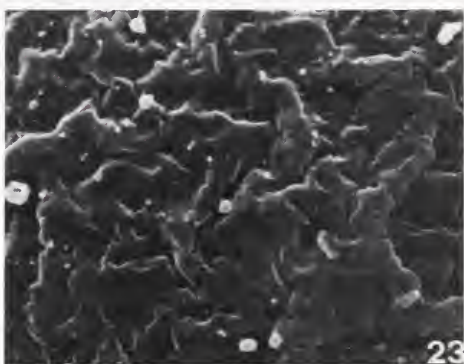
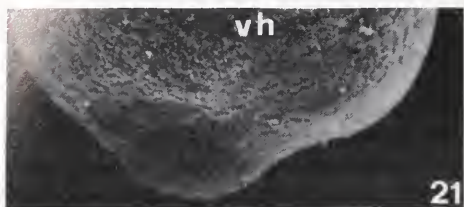
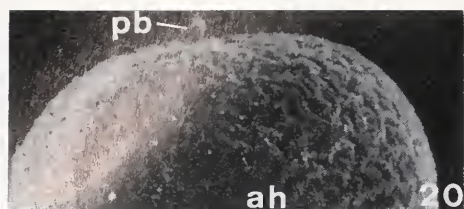
To understand their role in development, egg envelopes of many ascidian species have been subjects of extensive morphological and biochemical studies. However, previous SEM studies of egg envelopes were performed only on a few species including *Ascidella aspersa* (Mansueto and Villa, 1983), *Ciona intestinalis* (Bates, 1980; De Santis *et al.*, 1980) and *Phallusia mammillata* (Honnegger, 1982, 1986). The general morphological characteristics of *Ascidia malaca* egg envelopes are similar to those of the aforementioned species.

However, our study has detected some peculiarities of the test and follicle cells. The test cell membrane forms pseudopodia and invaginations, which probably reflect dynamic cell-to-cell contact, while the follicle cell membrane shows a reticulated lace-like structure and clefts in the basal region (previously observed only in *Ciona intestinalis*, Bates 1980). The size of these clefts might be involved in the ascidian "sperm reaction" (described by Lambert, 1982): loss of the mitochondrion would reduce the diameter of the spermatozoon to a size allowing easy penetration. Moreover, only intact spermatozoa have been detected on the plasma membrane of dechorionated eggs.

The morphological features of the surface of the dechorionated egg differ greatly before and after fertilization.

A smooth area around the polar pit marks a polarity in the unfertilized egg.

FIGURES 17–19. SEM of eggs fixed 3 min after fertilization showing shape modification (Fig. 17), different distribution of microvilli in equatorial area: (A) upper vegetal hemisphere (B) and lower vegetal hemisphere (C) (Fig. 18), and marked polarity of the folded areas (Fig. 19). Arrows indicate stumpy microvilli; arrowheads indicate slender microvilli. Figure 17, 700 $\times$. Figure 18, 4350 $\times$. Figure 19, 700 $\times$.



FIGURES 20–24. SEM of eggs fixed 7 min after fertilization showing changes in surface morphology. Figure 20: Supraequatorial area. ah = animal hemisphere; pb = polar body. 620X. Figure 21: Subequatorial area with a lobe formation at the pole. vh = vegetal hemisphere. 620X. Figure 22: View of the vegetal

After sperm penetration and between the ejection of the first polar body and the second maturation division, egg morphology changes drastically. In addition to the known shape deformations which may be caused by a cortical contraction during ooplasmic segregation (Ortolani, 1955; Reverberi, 1971; Sawada and Osanai, 1981), other fine modifications of the surface architecture occur.

In the newly fertilized egg (3 min), the mildly undulated animal area expands over the whole hemisphere as far as the subequatorial zone; the remaining vegetal part is corrugated. This rearrangement corresponds with the displacement of the chalk granules observed with the light microscope: divergent at the animal pole and convergent at the vegetal pole (Ortolani, 1955). These membrane modifications cause the test cells to migrate downwards and accumulate in the lower part of the vegetal region, as described by Conklin (1905).

Changes in the organization and distribution of the microvilli also occur at this time. In addition to the short type found in the unfertilized egg, two new types of microvilli appear: large stumpy microvilli in the equatorial area and slender ones condensed in the vegetal hemisphere, probably where the myoplasm accumulates.

After the ejection of the first polar body (7 min after sperm penetration) an apparent transposition of the features of the two halves is observed. The animal region becomes more undulated and is now densely covered by short microvilli. This occurs almost simultaneously with the disappearance of the polar pit. The vegetal region is less undulated since the egg elongates again, forming a lobe at the pole. This is followed by the ejection of the second polar body. According to the Jeffery and Meier model (1983) the protrusion is formed by a tight contractile ring of actin filaments that seems to push the endoplasm towards the animal pole creating a myoplasmic lobe in the vegetal pole. In the egg which has regained the spherical shape, microvilli concentrate to form a protuberance at the end of the vegetal hemisphere. Supernumerary spermatozoa begin to collect in this region. In fact, the spermatozoa follow the surface movements towards the vegetal pole, as do test cells (Conklin, 1905), chalk granules (Ortolani, 1955), and lectins (Monroy *et al.*, 1973; O'Dell *et al.*, 1974; Ortolani *et al.*, 1977; Zalokar, 1980). The migration of these external components appears to be coordinated with that of the myoplasm with which pigment granules, mitochondria, ribosome-like granules, subcortical granules, and filamentous structures cosegregate (Conklin, 1905; Reverberi, 1956; Berg and Humphreys, 1960; Mancuso, 1964; Sawada and Osanai, 1981; Jeffery and Meier, 1983).

When the egg is mature it is again spherical. Its surface is undulated primarily in the animal region and exhibits an almost homogeneous distribution of elongated microvilli; a small clump of spermatozoa stays at the vegetal pole.

We did not observe development after the fusion of the pronuclei began. We suggest that the elongation and diffusion of the microvilli might constitute a reserve of plasma membrane for the two cell stage. In *Halocynthia roretzi* (Satoh and Deno,

hemisphere showing the vegetal pole covered by clumped microvilli (asterisk). 620 $\times$. Figure 23: Magnification of the equatorial area showing stumpy microvilli. 3300 $\times$. Figure 24: Magnification of the clumped microvilli at the vegetal pole. 2550 $\times$.

FIGURES 25–28. SEM of eggs fixed 30 min after fertilization. Figure 25: Distribution of folded areas in animal and vegetal hemispheres; the vegetal pole is marked by clumping of supernumerary spermatozoa (asterisk). 580 $\times$. Figure 26: View of the vegetal hemisphere showing the polar zone covered by spermatozoa (asterisk). 580 $\times$. Figure 27: Magnification of elongated microvilli. 4730 $\times$. Figure 28: Magnification of the vegetal polar zone showing intact spermatozoa. 4980 $\times$.

1984) the appearance and disappearance of microvilli is associated with cleavage cycles.

Disappearance, concentration, and elongation of the microvilli in different regions of the activated egg could be the expression of a dynamic condition of the plasma membrane. Not only can the microvilli be considered a reserve of membrane, but they could also reflect a reorganization of the cell surface on which cytoplasmic events depend.

Changes in density, distribution, and organization of the microvilli have been observed after fertilization in sea urchin (Eddy and Shapiro, 1976; Schroeder, 1979; Longo, 1986), fish (Iwamatsu, and Keino, 1978), mouse (Nicosia *et al.*, 1978), and amphibian eggs (Monroy and Baccetti, 1975; Charbonneau and Picheral, 1983). Local surface differentiation consisting of special microvilli occur on the polar lobes in the egg of some gastropods (Dohmen and Van der Mey, 1977); it is therefore suggested that a relationship exists between the surface structures and the localization or expression of the morphogenetic factors in the polar lobes.

In ascidian eggs the cell surface elements seem to be connected to the cytoskeleton; the presence in the cortex of a contractile actin-network which produces the force causing cytoplasmic movements has been demonstrated in *Styela*, *Boltenia*, and *Ciona* eggs (Jeffery and Meier, 1983, 1984; Sawada and Osanai, 1984, 1985). According to Jeffery (1984), ooplasmic segregation includes the movements of cell surface components, cytoskeleton, cytoplasmic organelles, and localized maternal mRNA molecules, which are associated in a cytoplasmic complex. These authors, therefore, proposed that the cortical contraction is the main cause of the polarized ooplasmic movements in the ascidian species.

Several other results from studies of *Ascidia malaca* and *Phallusia mammillata* eggs suggest that a pattern of developmental information is localized in the plasma membrane; an early surface specialization which reflects a cytoplasmic compartmentalization of morphogens may also exist (Monroy *et al.*, 1973; O'Dell *et al.*, 1974; Ortolani *et al.*, 1977; Zolotar, 1980). The lack of external K^+ ions affects cellular activities by acting directly on the membrane of the unfertilized egg; the probable rearrangement of the membrane structures provokes, among other things, a change in the detectability of Con A binding sites (Di Pisa *et al.*, 1982). The role of the plasma membrane during embryonic development has been demonstrated by the differentiation of neural structures and of tissue-specific enzyme obtained through trypsin induction (Ortolani *et al.*, 1979).

Based on these considerations we suggest that in ascidian eggs the cell membrane components and related topographic changes could be the first cause of the ooplasmic movements, which are driven by the contractile actin-network connected to the plasma membrane; therefore, ascidian ooplasmic segregation could depend on surface reorganization.

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THE GREEN HYDRA SYMBIOSIS: ANALYSIS OF A FIELD POPULATION

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ABSTRACT

Green hydra were abundant on the alga *Vaucheria taylorii* in a shallow woodland stream near Lincoln, Nebraska, from March to June 1985. Green hydra were also found in low numbers throughout the winter. The algal-animal biomass characteristics of field populations of green hydra are compared to those of cultures established from the field populations and maintained under defined laboratory conditions. Although of similar protein biomass, freshly collected hydra contained greater numbers of symbiotic algae than did cultured hydra. Algae in field hydra were larger and contained more chlorophyll than algae in cultured hydra. Field populations of green hydra were highly productive; $16 \mu\text{g C} \cdot \text{h}^{-1} \cdot \text{mg hydra protein}^{-1}$ were fixed by the endosymbiotic algae at an irradiance of $28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

INTRODUCTION

The green hydra-*Chlorella* association has been intensively studied for two decades. The association involves *Chlorella*-like algae living within the digestive cells of the freshwater polyp *Hydra viridissima*. It is one of a group of algal-invertebrate associations called phycozoans (Pardy, 1983) to emphasize their algal-animal composite nature. Much is known about the metabolic interdependency of the bionts, the recognition processes whereby hydra acquire algae, and some of the regulatory processes which stabilize the association. Comprehensive reviews concerning the association may be found in Cook (1980, 1981, 1983).

The majority of experimental studies have used laboratory cultures of green hydra. These hydra have been cultured under defined conditions with respect to temperature, light intensity, and feeding and maintenance schedules. Such stringent culture methods yield populations of green hydra of uniform size and age distributions, with stable densities of symbiotic algae. Although these laboratory cultures of green hydra are useful for certain experimental studies, they may bear little resemblance to green hydra living in ponds and streams. While a variety of ecological studies (Welch and Loomis, 1924; Miller, 1936; Bryden, 1952; Carrick, 1956; Cuker and Mozley, 1981; Ribi *et al.*, 1985) have attempted to describe the seasonal distribution and abundance of non-symbiotic hydra, similar studies of green hydra are lacking. Although green hydra have been collected from a variety of habitats [standing water (Whitney, 1907; Lashley, 1915), a swamp (Carrick, 1956), a river (McAuley, 1984), and roadside ditches (Forrest, 1959)], none of these studies have described the algal-animal biomass characteristics of freshly collected hydra.

In this paper we analyze the biomass parameters of symbiotic algae in field populations of green hydra, and compare these parameters to those of cultures established

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from the field populations and maintained under defined laboratory conditions. We also describe the seasonal variation in algal-animal biomass parameters of green hydra, and provide some estimates of the productivity of field populations. The results show that field populations of green hydra contain greater numbers of algae and are far more productive than hitherto measured in laboratory-cultured green hydra.

MATERIALS AND METHODS

Location of study

A small stream near a large reservoir (Pawnee Reservoir) 10 miles northwest of Lincoln, Nebraska (40°50'37"N, 96°51'37"W) was chosen as a study site, as green hydra were known to occur in this stream (Pardy and Glider, 1984). The spring-fed stream (Pawnee stream) extended 1.5 km above the study site. There were two small ponds upstream from the site, one 122 m long and the other 50 m long. At the study site the water depth varied from 15 cm to 60 cm, depending on local rainfall. The bottom was muddy and often filled with litter from overhanging trees.

Collection and maintenance of hydra

Leaf litter and attached submerged vegetation were collected from the bottom of Pawnee stream; floating *Lemna* sp. plants were collected when present. As hydra were initially found in great abundance on algal mats of the filamentous chrysophyte *Vaucheria taylorii*, these mats were collected on a regular basis and examined for green hydra. Hydra were routinely sampled by removing four to five mats, about 200 cm² each, of *Vaucheria taylorii* and placing these into separate containers. In the laboratory, green hydra detached from the algal filaments and were readily collected with a Pasteur pipette. All analyses on hydra were performed within 24 h after collection.

To compare the biomass parameters of freshly collected hydra (=field hydra) with those of hydra maintained under defined conditions in the laboratory, hydra collected in March 1985 were brought into culture. These hydra were slowly acclimated to M solution (Muscantine and Lenhoff, 1965) by gradually replacing streamwater with M solution. Cultures were then maintained under continuous light at two irradiances (5 and 30 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 21°C and fed three times each week with freshly hatched *Artemia* nauplii. The culture medium was replaced daily; twice daily on feeding days. After 15 months of laboratory culture, population growth rates of Pawnee hydra maintained at 5 and 30 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ were measured as described by Muller-Parker and Pardy (1987). Population growth rate constants and doubling times were calculated according to Loomis (1954).

Ultrastructure of symbiotic algae

Transmission electron microscopy was used to examine the ultrastructure of symbiotic algae in freshly collected hydra. Hydra collected on March 18, 1985 were cut into pieces in phosphate buffered (0.1 M, pH 6.8) 3% glutaraldehyde at room temperature. The segments were fixed for 2 h in the glutaraldehyde fixative, then post-fixed with 1% OsO₄ in buffer for 1 h. Following dehydration in a graded series of ethanol, specimens were embedded in Epon 812. Sections stained in 2% aqueous uranyl acetate and lead citrate were viewed in a Philips 201 electron microscope operated at 60 kv.

Hydra biomass parameters

Algal and animal biomass parameters (protein, number of algae per μg hydra protein, number of algae per hydra digestive cell, algal chlorophyll and algal cell volumes) were measured on pooled samples of 100 hydra using previously described procedures (Muller-Parker and Pardy, 1987), with the exception that from 5 to 25 hydra were pooled to measure the number of algae per digestive cell. Biomass parameters of laboratory cultures of Pawnee hydra were measured after 15 months of culture. These hydra were last fed 48 hours before analysis.

Productivity of field hydra

The productivity of Pawnee hydra collected on April 3 and June 3, 1985 was measured in the laboratory. Carbon fixation at various irradiances was measured 24 h after collection. Groups of 25 hydra, each with one bud for uniformity, were incubated with $\text{NaH}^{14}\text{CO}_3$ ($0.8 \mu\text{Ci} \cdot \text{ml}^{-1}$) in 5.0 ml of filtered ($>0.45 \mu\text{m}$) streamwater supplemented with 5 mM NaHCO_3 in glass beakers covered with various layers of screen. Replicate groups of hydra were incubated in the dark to correct for any dark fixation of $\text{NaH}^{14}\text{CO}_3$. Incubation media were sampled at the start of the experiments for total ^{14}C activity. At the end of the one-hour incubations hydra were thoroughly rinsed in cold, filtered streamwater and then homogenized in distilled water. Organic ^{14}C retained by hydra, protein biomass, and numbers of algae were determined as previously described (Muller-Parker and Pardy, 1987). Total CO_2 in filtered streamwater was calculated from the total alkalinity—measured potentiometrically (Golterman, 1969)—of samples of filtered streamwater collected on July 17, 1986. After correction for dark fixation, the amounts of ^{14}C retained by hydra tissues were converted to rates of carbon fixation (Vollenweider, 1969) normalized to hydra protein biomass and to numbers of algae.

RESULTS

Distribution of hydra in Pawnee stream

The distribution of green hydra in Pawnee stream from March 1985 to May 1986 was highly variable and appeared related to the presence of the filamentous chrysophyte alga, *Vaucheria taylorii* (Blum, 1971). Green hydra on *V. taylorii* were not immediately obvious, as the slender polyps and color of hydra bore remarkable resemblance to the algal filaments. Other samples of submerged vegetation yielded few hydra although individuals were occasionally found anchored to *Lemna* leaves as found by Pardy and Glider (1984).

Specimens of *Vaucheria taylorii* were identified by the characteristic structure of the antheridia and oogonia (Blum, 1971). These reproductive structures developed frequently in algae maintained in the laboratory at 21°C under continuous light. Thus it was possible to verify the taxonomic identity of this alga from various collections made throughout the year. This alga (formerly named *Vaucheria geminata* var. *racemosa*) has been reported from creeks around Lincoln, Nebraska (Saunders, 1894). *V. taylorii* was common in shallow, unshaded areas of the stream where there was slow water flow. The depth of the water above algal mats varied from 1 to 12 cm.

Peak abundances of field hydra averaged about 500 hydra per 200 cm^2 mat of *V. taylorii*, and occurred from March to June 1985. From June to September 1985 the stream occasionally dried up and did not contain aquatic vegetation or hydra. The site was not visited until January 1986, at which time *V. taylorii* was abundant and



FIGURE 1. Algal symbiont in green hydra freshly collected from Pawnee stream. Scale bar = 1 μm .

hydra were found in low densities (1–10 individuals per 200 cm^2 mat). Hydra persisted on *V. taylorii* throughout the winter and spring of 1986. At all times, algal mats contained large numbers of zooplankton and the hydra were frequently observed to feed on these. Large nonsymbiotic brown hydra were often found among the green hydra, but never in great abundance.

All green hydra collected in March and April 1985 were asexual. Many had from one to five buds per individual. By early May, half of the hydra were sexual, bearing both ovaries and testes. Ninety-five percent of the collected hydra had gonads in late May. In early June the number of hydra bearing gonads had decreased to 50%; most of these bore testes only. Embryonic thecae resembled those described by McAuley (1984) for *Hydra viridissima*. A two-chambered theca, characteristic of *Chlorohydra hadleyi* (Forrest, 1959), was never seen. Released eggs did not develop under laboratory conditions.

Morphology, size, and chlorophyll content of algal symbionts from green hydra

The algal symbiont of hydra collected from Pawnee stream resembles the algae found in the English strain (Pardy, 1976; Jolley and Smith, 1978) of green hydra in that it possesses a pyrenoid traversed by a single thylakoid (Fig. 1).

The size of algae in field and cultured populations of green hydra is given in Table

TABLE I

Size of symbiotic algae in green hydra either (a) freshly collected or (b) maintained in culture for 15 months

	Algal diameter (μm)	Algal cell volume (μm^3)
(a) March 17, 1986	4.38 (± 0.93) ^a	44
May 7, 1986	3.44 (± 0.85)	21
(b) Low light ($5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	2.90 (± 0.55)	13
High light ($30 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	2.59 (± 0.41)	9

^a $\pm$ SD; n = 100.

I. Algae varied greatly in size in hydra collected on two dates in 1986, but cell volumes of algae in freshly collected hydra were at least twice those of algae in cultured green hydra (Table I).

Algae in field hydra contained more than four times the amount of chlorophyll measured in algae from cultured hydra (Table II). Although the chlorophyll content of algae varied greatly between cultured and field hydra, the ratio of chlorophyll *a* to chlorophyll *b* was about one in algae obtained from all hydra (Table II).

Algal-animal biomass parameters of green hydra

Algal numbers in hydra are readily obtained by counting the number of algae in dissociated digestive cells. Figure 2 shows the variation in numbers of algae in digestive cells in hydra collected in 1985 and 1986 from Pawnee stream. The average number of algae per cell ranged from 14 (March 1986) to 34 (June 1985). Ambient stream temperatures are also given in Figure 2. There appears to be no relationship between algal densities in green hydra and water temperature. Hydra collected in near-freezing waters (January and February 1986) under substantial layers of ice and

TABLE II

Chlorophyll content of algae isolated from green hydra either (a) freshly collected in 1985 or (b) maintained in culture for 15 months

	Total Chl $\cdot$ cell ⁻¹ (pg)	Chl <i>a</i> $\cdot$ cell ⁻¹ (pg)	Chl <i>b</i> $\cdot$ cell ⁻¹ (pg)	<i>a/b</i>
(a) March 18	0.63	0.32	0.31	1.01
March 20	0.80	0.44	0.36	1.20
April 3	0.68	0.35	0.33	1.06
May 7	0.77	0.40	0.37	1.05
May 22	0.45	0.21	0.23	0.92
June 3	0.49	0.28	0.21	1.34
(b) Low light ($5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	0.15 (± 0.02) ^a	0.08 (± 0.01)	0.07 (± 0.01)	1.14 (± 0.06)
High light ($30 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	0.04 (± 0.01)	0.02 (± 0.004)	0.02 (± 0.006)	1.16 (± 0.24)

^a $\pm$ SD; n = 5.

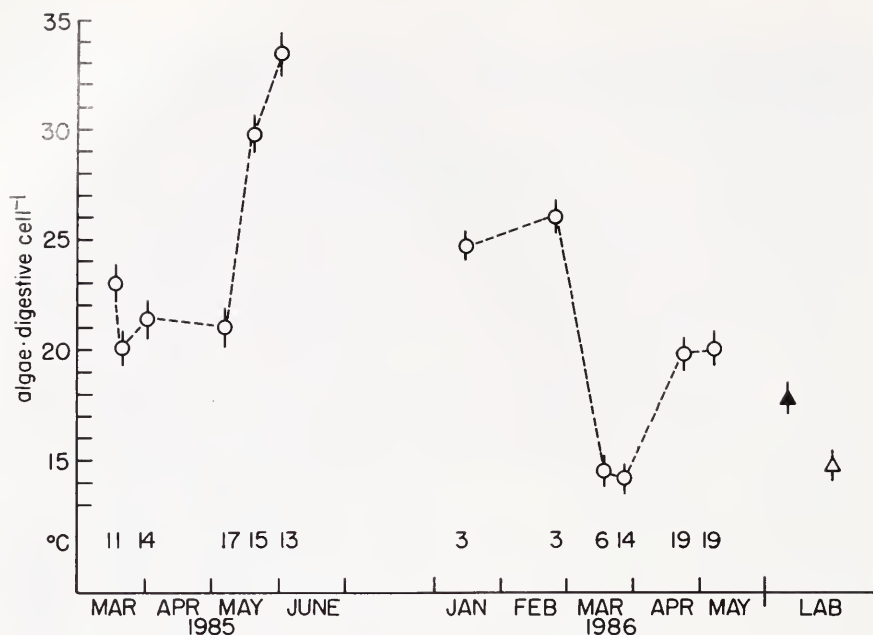


FIGURE 2. Number of algae per digestive cell (open circles, $\pm$ SE) in hydra collected from Pawnee stream in 1985 and 1986. Ambient streamwater temperatures measured on the collection dates are included near the origin of the y-axis. For comparison, the mean numbers of algae per digestive cell of cultured Pawnee hydra maintained at $5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (solid triangles) and at $30 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (open triangles) are included.

snow contained a full complement of algae (Fig. 2). The numbers of algae per digestive cell of cultured hydra are included in Figure 2 for comparison. Digestive cells of cultured hydra contained under 20 algae, whereas 50 or more algae per digestive cell were frequently counted in freshly collected green hydra. In general, digestive cells of field populations of these green hydra contained greater numbers of symbiotic algae than those of hydra maintained in laboratory culture.

The density of symbiotic algae in freshly collected hydra was also measured by determining the number of algae per μg hydra protein in 1985 (Fig. 3a). Algal densities varied two-fold over a four month period; algal densities measured in June were double those obtained in March. This change in algal density coincided with a shift from a predominantly asexual population to a predominantly sexual population. Algal densities in cultured hydra were within the range obtained for field hydra; hydra maintained at a low irradiance contained more algae than those maintained at a high irradiance (Fig. 3a).

The size of field hydra was estimated by measuring the weight of protein per hydra individual. Figure 3b shows that the protein content of field hydra ranged from 2 to $8 \mu\text{g}$ protein, and that the increase in algal density (Fig. 3a) was accompanied by a decrease in protein content. Thus, hydra collected in June 1985 were smaller and contained higher densities of algae than those collected in March 1985.

Laboratory cultures of Pawnee hydra contained high protein biomass and low algal densities in comparison to field populations (Fig. 3a, b). Hydra maintained at $5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ were larger and contained more algae than individuals maintained at 30

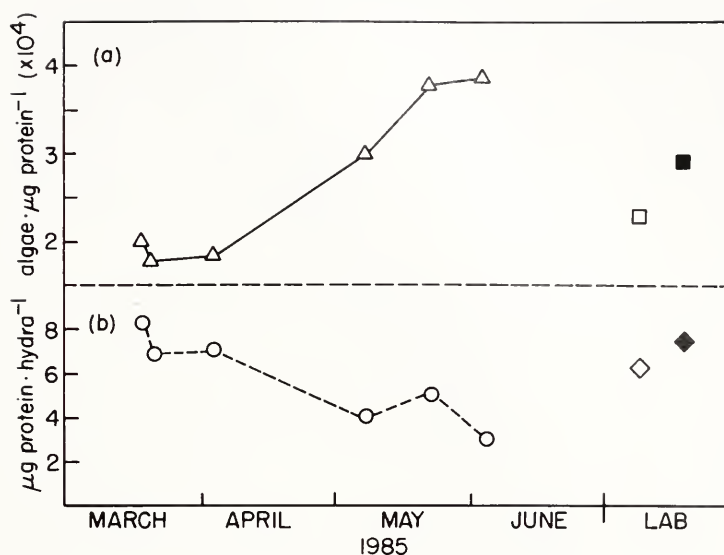


FIGURE 3. (a) Number of algae per μg hydra protein in hydra collected from Pawnee stream in 1985 (open triangles). (b) Protein biomass of hydra collected from Pawnee stream in 1985 (open circles). Parameters obtained in cultured hydra (Lab) maintained at $5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (solid symbols) and at $30 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (open symbols) are included to the right of the field data.

$\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Population growth rate constants of Pawnee hydra maintained at both irradiance levels were the same and averaged 0.28 day^{-1} , which resulted in a doubling time of 2.5 days.

Productivity of field hydra

The productivity of green hydra collected from Pawnee stream on April 3, 1985 and on June 3, 1985 was measured in the laboratory. The amount of carbon fixed by these hydra increased linearly with increase in irradiance from 0 to $30 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Fig. 4). Photosynthetic efficiencies (the slopes of the lines in Fig. 4) were normalized to protein biomass and number of algae. Photosynthetic efficiencies of April hydra were $0.45 \mu\text{g C} \cdot (\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{ protein}$ and $0.022 \mu\text{g C} \cdot (\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1} \cdot \text{h}^{-1} \cdot 10^6 \text{ algae}^{-1}$, whereas those of June hydra were $0.60 \mu\text{g C} \cdot (\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{ protein}$ and $0.016 \mu\text{g C} \cdot (\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1})^{-1} \cdot \text{h}^{-1} \cdot 10^6 \text{ algae}^{-1}$. Hydra collected on these two dates differed greatly in algal density (Fig. 3a) and in protein biomass (Fig. 3b). The hydra collected on April 3 contained twice the amount of protein and half the number of algae as hydra collected on June 3, 1985. Figure 4a shows that on a protein basis the smaller hydra with greater numbers of algae (June hydra) were more productive than the April hydra. When the data were normalized to numbers of algae, hydra with low densities (April hydra) were more productive on a cell basis (Fig. 4b). These results suggest that although high algal densities result in greater productivity on a unit biomass basis, the amount of carbon fixed per alga is greatly reduced.

DISCUSSION

We have shown that field populations of symbiotic hydra are highly productive and maintain high densities of algae throughout the year. There may be significant

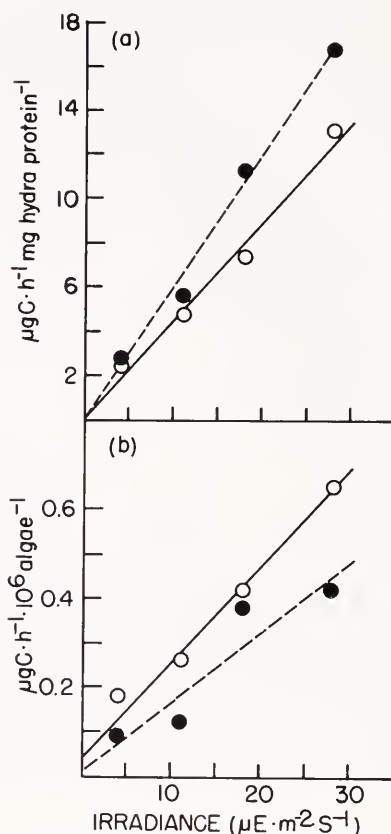


FIGURE 4. Productivity of field hydra at different irradiances. Rates were normalized to (a) protein biomass and (b) numbers of algae. Open circles represent production rates of hydra in April 1985 and closed circles are those obtained for hydra in June 1985.

differences in the algal and animal biomass parameters and productivity of field and laboratory populations of green hydra. Symbiotic algae isolated from field populations of green hydra are larger and contain more chlorophyll than those isolated from cultured hydra. When maintained under controlled laboratory conditions, the algal-animal biomass parameters and growth rates of these hydra resemble those of the Florida hydra strain (Muller-Parker and Pardy, 1987) kept under the same conditions.

Algal-animal biomass parameters

The biomass parameters of green hydra collected in 1985 and 1986 from one site were quite variable. Variation in protein biomass of hydra, in numbers of algae, and size and chlorophyll content of the algae can result from several processes which are not mutually exclusive. Changes in the physical environment (light, temperature, water flow) and in prey availability may affect these parameters. Genetic differences in hydra populations collected at different times may be important (McAuley, 1984). The relative influence of these factors cannot be presently assessed.

Hydra were found in Pawnee stream throughout the winter. Others have found both symbiotic hydra (Whitney, 1907) and non-symbiotic hydra (Carrick, 1956) in water of very low temperatures. However this is the first report which shows that green hydra maintain high densities of symbiotic algae throughout the winter.

There was a great increase in algal density and decrease in protein biomass of field hydra during the spring of 1985 (Fig. 3a, b). At the same time these hydra shifted from a predominantly asexual population to a sexual population. This suggests that sexual individuals may contain higher densities of algae than non-sexual hydra. This needs to be confirmed in hydra maintained under controlled conditions in the laboratory.

The most striking difference between the algal-animal biomass parameters of freshly collected and cultured Pawnee hydra was the large difference in algal cell volume (Table I) and chlorophyll content of the algae (Table II). The large size of the algae and the great number of algae per digestive cell (Fig. 2) in freshly collected hydra show that these hydra contain a proportionately greater ratio of plant to animal biomass than cultured hydra. This suggests that regulatory processes governing the number of algae in hydra cells may be substantially different under field conditions.

Hydra collected from Pawnee stream and maintained in culture for 15 months showed similar responses to light as the Florida strain of hydra maintained under the same conditions (Muller-Parker and Pardy, 1987). In both Pawnee and Florida hydra, protein biomass and algal densities decreased with increase in culture irradiance, whereas population growth rates were unaffected by irradiance. An increase in algal cell volume and decrease in chlorophyll per alga occurred in both Pawnee and Florida hydra with increase in culture irradiance. These results suggest that green hydra from different localities respond in a consistent manner to changes in culture irradiance.

Pawnee hydra were smaller than Florida hydra as the protein biomass of cultured Pawnee hydra maintained at 5 and 30 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (7.6 and 6.3 μg) was less than that of Florida hydra maintained at the same irradiances (11.0 and 9.5 μg ; Muller-Parker and Pardy, 1987). The population growth rates of Pawnee hydra were slightly higher than those of Florida hydra; population doubling times for Pawnee hydra were 2.5 days whereas those of Florida hydra averaged about 3 days. Algal cell volume and the number of algae per digestive cell were similar for both Pawnee and Florida hydra.

Productivity of field collected Pawnee hydra

The productivity of hydra collected from Pawnee stream on two dates was high; rates of carbon fixation were over four times as great as those obtained with cultured Florida hydra incubated under similar conditions (Muller-Parker and Pardy, 1987). Although field Pawnee hydra and cultured Florida hydra are not strictly comparable, the productivity of cultured Pawnee hydra was not measured in this study. The previous light history may affect photosynthetic rates, as photosynthetic efficiencies derived for Pawnee hydra were greater than those obtained for cultured Florida hydra (Muller-Parker and Pardy, 1987). Differences in productivity cannot be attributed to differences in protein biomass and density of algae of field and laboratory hydra, since Pawnee hydra collected in April and June 1985 were substantially different in these two parameters. However, algal cell size and chlorophyll content of algae were much greater in freshly collected Pawnee hydra than in cultured Florida hydra, which may account for the high productivity of Pawnee hydra.

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ZOOPLANKTON FEEDING ECOLOGY: CONTENTS OF FECAL PELLETS OF THE COPEPOD *CENTROPAGES VELIFICATUS* FROM WATERS NEAR THE MOUTH OF THE MISSISSIPPI RIVER

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ABSTRACT

The *in situ* diet of the copepod *Centropages velificatus* was investigated using scanning electron microscopy. Contents of fecal pellets produced upon natural diets were compared with assemblages of available phytoplankton. Samples came from continental shelf waters near the mouth of the Mississippi River. A wide variety of particle sizes and types were ingested. The dominant phytoplankton taxa in pellets sometimes mirrored those dominant in the water, particularly if these were large solitary cells or elongate chains of smaller cells. However, in other cases the dominant phytoplankton remains in pellets were those of cells that were not abundant in the water. Crustacean remains and fine particles of silt from the river plume were also frequent dominant components. *C. velificatus* is clearly an omnivore.

The often poor correspondence between ingested and abundant phytoplankton taxa and the frequency of crustacean remains suggests that this copepod spends much of its time feeding as a raptorial carnivore upon other zooplankton.

INTRODUCTION

Omnivorous feeding is common in planktonic marine copepods. Numerous species ingest both phytoplankton and zooplankton (reviewed by Turner, 1984a), and some large copepods can eat larval fish (Turner *et al.*, 1985 and references therein). However, most studies of copepod omnivory have used laboratory diets such as cultured phytoplankton and zooplankton. Examinations of the diets of omnivorous copepods feeding upon natural food assemblages have been less frequent.

As part of a study of planktonic food webs that support larval fish in northern Gulf of Mexico continental shelf waters, an effort has been made to define diets of copepod species that are prey of fish larvae (Turner, 1984b, c, d, 1985, 1986a, 1986b). Contents of fecal pellets produced from food ingested prior to capture have been compared with co-occurring phytoplankton species assemblages. A general pattern that has emerged for many calanoid species is that of indiscriminant suspension feeding; phytoplankton taxa in fecal pellets reflect the composition of the natural phytoplankton. However, certain copepods such as the large calanoid *Labidocera aestiva* (Turner, 1984c) or several cyclopoid species (Turner, 1986b) did not exhibit this pattern as strongly as other typically herbivorous species. The fecal pellets of *L. aestiva* and the cyclopoids contained considerable crustacean remains, and disproportionately high abundances of large phytoplankton cells that were not particularly abundant in the water. These results suggest that these copepods are primarily raptorial omnivores, feeding frequently on other animals and large phytoplankton cells.

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In addition to the largely qualitative fecal pellet studies, we have performed quantitative grazing studies on three species of calanoid copepods: *Acartia tonsa*, *Eucalanus pileatus*, and *Centropages velificatus* (Turner and Tester, 1986; Tester and Turner, 1986). Although *A. tonsa* and *E. pileatus* exhibited nonselective grazing, with phytoplankton taxa being ingested in proportion to their abundance, this pattern did not hold for *C. velificatus*. Regression coefficients between rates of ingestion of individual phytoplankton taxa and abundance of those taxa, or between total phytoplankton ingestion rate and total phytoplankton abundance were low. This suggested that *C. velificatus* was feeding primarily as a carnivore, a detritivore, or as a selective herbivore upon less-abundant phytoplankton. Accordingly, the contents of *C. velificatus* fecal pellets produced at these and other northern Gulf of Mexico stations were examined with the scanning electron microscope (SEM).

Centropages velificatus Oliveira is widely distributed throughout the tropical and subtropical western Atlantic and Gulf of Mexico (Fleminger and Hulsemann, 1973). This species is the Atlantic cognate of the Indo-Pacific species *Centropages furcatus* Dana (Fleminger and Hulsemann, 1973). Thus, numerous Atlantic and Gulf of Mexico records for *C. furcatus* (Owre and Foyo, 1967 and references therein; Bowman, 1971; Paffenhöfer and Knowles, 1980) apply to *C. velificatus*. This copepod is one of the numerically dominant mesozooplanktonic calanoids in continental shelf waters of the northern Gulf of Mexico (Marum, 1979; Minello, 1980), and it is consumed in these waters by several species of fish larvae (Govoni *et al.*, 1983).

Various *Centropages* species have long been known to be omnivores, ingesting both cultured phytoplankton, *Artemia* nauplii, or cultured copepod nauplii (reviewed by Turner, 1984a). Paffenhöfer and Knowles (1980) found that *C. velificatus* from the southeastern United States continental shelf ingested a higher percentage of its body nitrogen in the form of laboratory-reared copepod nauplii (*Pseudodiaptomus coronatus*) than as large diatoms of the genus *Rhizosolenia*. However, the temperate congener *C. hamatus* ingested a higher percentage of its body carbon as natural phytoplankton than as field-caught copepod nauplii (mostly *Acartia* spp.) (Conley and Turner, 1985). In addition, *C. typicus* has been shown to ingest yolk-sac larvae of two fish species (Turner *et al.*, 1985).

Results presented here support previous *Centropages* feeding studies, as well as the suggestion from our quantitative grazing data (Turner and Tester, 1986; Tester and Turner, 1986) that *C. velificatus* is a raptorial omnivore, feeding to a great extent upon large phytoplankton cells and other crustaceans. This is, to my knowledge, the first study of the *in situ* feeding habits of this copepod.

MATERIALS AND METHODS

Samples were collected from ten stations (Table I) on three cruises in 1982 and 1983. Surface water samples (500 ml) for phytoplankton analyses were preserved in Utermöhl's solution (Guillard, 1973) immediately prior to zooplankton tows. Surface tows with 363 μm mesh nets were used for copepod collection. The mesh was coarse enough to allow passage of all phytoplankton and microzooplankton, thereby preventing possible net feeding. Copepods were immediately sorted by pipette and isolated in surface seawater within 5–15 min of collection. After pellets reflecting gut contents upon capture had been produced (usually < 0.5 h), they were individually removed by pipette, placed in a mixture of filtered seawater and 20 μm -mesh-screened surface water (containing natural microbes), and left for 24–36 h at approximately 20°C for microbial stripping of pellet peritrophic membranes, which if left intact, mask contents (Turner, 1978; 1979). Pellets were preserved in 5% formalin: seawater solutions for analyses ashore.

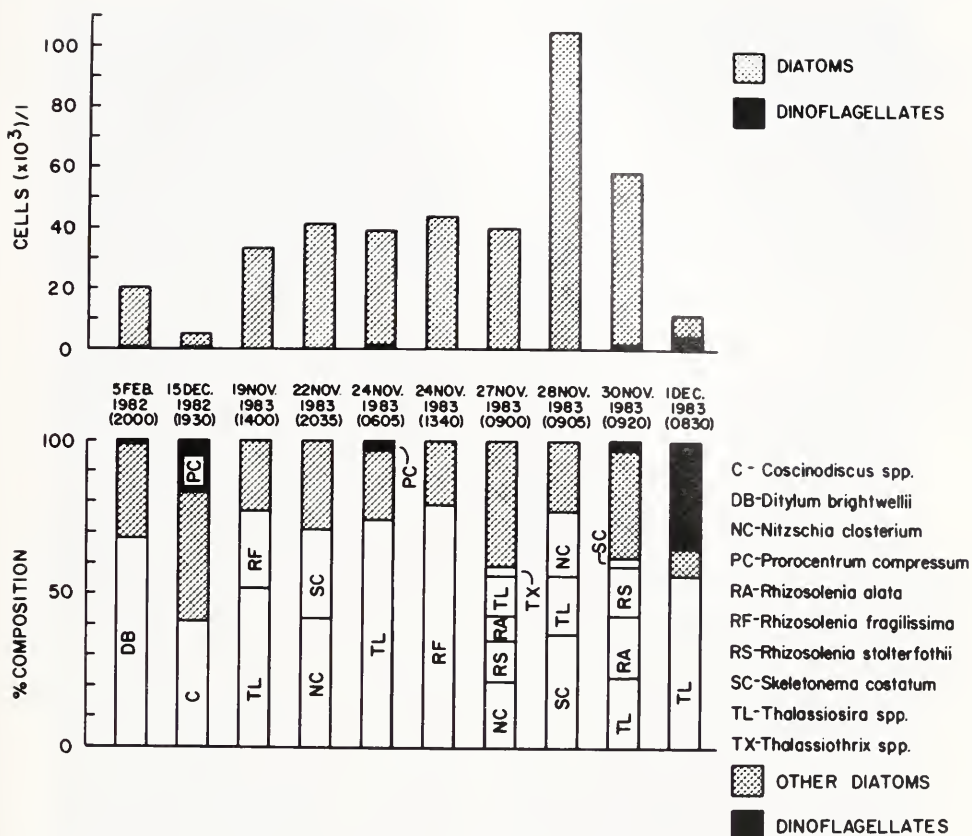


FIGURE 1. Abundance (upper) and composition (lower) of natural phytoplankton assemblages in surface waters at sampling stations.

Preserved pellets were individually sorted by pipette and drawn onto Whatman* GFC glass fiber filters. Filters with attached pellets were washed in distilled water for salt elimination, dehydrated in a graded ethanol series, critical point dried, coated with gold:palladium, and examined with a ISI-30 SEM at 15 kV.

All fecal pellets were from *Centropages velificatus* adult females. One hundred twenty-two (122) micrographs were taken from 38 fecal pellets. SEM examination preceded phytoplankton analyses to avoid possible bias in characterization of pellet contents. Entire visible sides of each pellet were examined. The 26 micrographs presented here (21% of those taken) are representative of pellet contents. Phytoplankton analyses were made with the Utermohl inverted microscope technique (Lund *et al.*, 1958).

RESULTS

Abundance and species composition of surface phytoplankton varied considerably (Fig. 1). Abundance ranged from $5.4\text{--}105.1 \times 10^3$ cells/l. With the exception of

* Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

TABLE I

Locations, times, and surface salinities of sampling stations

Date	Local time	Latitude (N)	Longitude (W)	Surface salinity (‰)
5 Feb. 1982	2000	28°53'	89°29'	21.5*
15 Dec. 1982	1930	28°54'	89°28'	18.1*
19 Nov. 1983	1400	28°52'	89°29'	—*
22 Nov. 1983	2035	28°50'	98°30'	31.5
24 Nov. 1983	0605	28°53'	89°29'	31.8
24 Nov. 1983	1340	28°51'	89°25'	29.6*
27 Nov. 1983	0900	29°02'	89°30'	26.1
28 Nov. 1983	0905	28°48'	89°57'	26.6
30 Nov. 1983	0920	28°48'	89°58'	30.7
1 Dec. 1983	0830	28°52'	89°29'	21.0*

* Stations with a high silt load.

two stations, assemblages were dominated by diatoms. However, on 15 December 1982, the dinoflagellate *Prorocentrum compressum* comprised 17% of total cells, and on 1 December 1983 a combination of the dinoflagellates *P. compressum*, *P. micans*, and *Gyrodinium* sp. comprised 35% of cells present. Abundant diatoms included large solitary cells such as *Ditylum brightwellii* (16.5 x 148.5 μ m), *Coscinodiscus* sp. (33–53 μ m diameter), and *Thalassiosira* sp. (13–36 μ m diameter). Also abundant were various chain-forming diatoms such as *Rhizosolenia fragilissima*, *R. stolterfothii*, and *Skeletonema costatum*.

In addition to phytoplankton, silt from the plume of the Mississippi River was abundant in surface waters from half of the stations (Table I).

Contents of fecal pellets varied widely among stations. At three stations from the river plume (5 February 1982, 19 November 1983, and 24 November 1983 at 1340 h) silt was the only component of fecal pellets (Figs. 2a, b, c), despite the presence of abundant phytoplankton (Fig. 1). Water samples from the station on 15 December 1982 also contained a heavy silt load, but fecal pellets contained primarily cells of the large (33 μ m diameter) dinoflagellate *Prorocentrum compressum* (Fig. 2d) in addition to silt. It is interesting that at this station *P. compressum* comprised only 11% of cells counted, whereas the similarly large (33–53 μ m diameter) diatom *Coscinodiscus radiatus* comprised 41% of cells counted, but no *C. radiatus* remains were observed in pellets. At other stations, however, large solitary centrics of the genus *Thalassiosira* (26–33 μ m diameter) were present in pellets as either intact cells or cell fragments (Figs. 2e, f, 3a, b, c, d). *Thalassiosira* abundance at these stations was high, with values of 35.7, 23.4, and 6.5×10^3 cells/l on 24 November, 28 November, and 1 December 1983, respectively, accounting for 74%, 19%, and 56% of cells on these dates. In addition to *Thalassiosira* spp., pellets from 24 November 1983 (0605 h) contained fragments of *P. compressum* cells (Fig. 3e) and remains of crustaceans (Fig. 3f). Crustacean remains, probably of copepod nauplii, were present in pellets from all remaining stations (Fig. 4c, d, 5d, 6f), along with remains of both small and large diatoms (Figs. 4a, b, 5a, b, 6a, b, c, d) and dinoflagellates (Figs. 5c, 6e). These three stations (22 November, 27 November, 30 November 1983) all had intermediate levels of phytoplankton abundance (Fig. 1), intermediate to high salinity, and reduced silt load (Table I). It is interesting that phytoplankton taxa of long linear dimension such as solitary *Thalassiothrix* sp. cells (up to 154 μ m long) or chains of *Skeletonema costatum* (maximum chain length unknown) were the most common phytoplankton

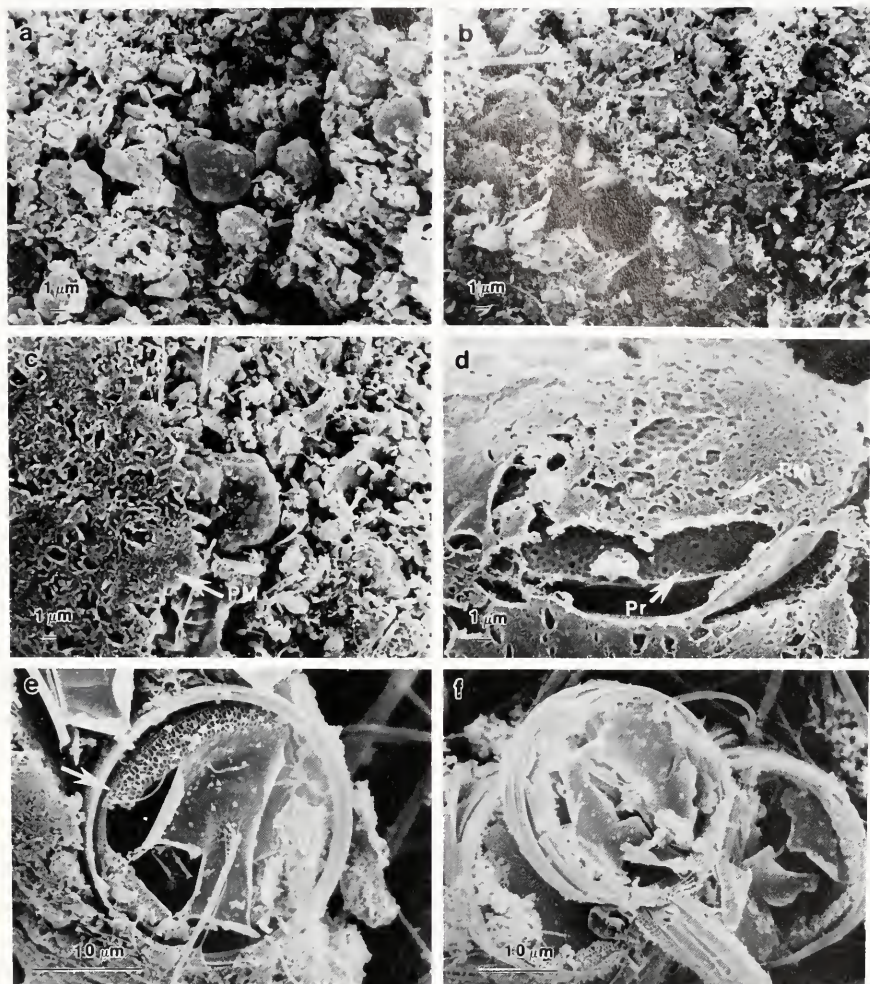


FIGURE 2. Contents of *Centropages velificatus* fecal pellets. a, 24 November 1983 (1340), b, 19 November 1983, c, 5 February 1982 (note peritrophic membrane, PM), d, 15 December 1982 (note peritrophic membrane, PM, and *Prorocentrum compressum* cell, Pr), e, 1 December 1983 (note *Thalassiosira* sp. cell at arrow), f, 28 November 1983 (note *Thalassiosira* sp. cells).

observed in pellets from 27 and 30 November (Figs. 5a, b, 6a, b), even though these taxa comprised only 3% each, respectively, of the cells in these samples. Small solitary diatom cells, though sporadically present in pellets (Fig. 4a, b, 6c), were never a dominant component.

DISCUSSION

Centropages velificatus is clearly an omnivore. Fecal pellets representing *in situ* feeding contained primarily the remains of other crustaceans and large or elongate phytoplankton taxa. These field results support previous laboratory studies with the same species (Paffenhöfer and Knowles, 1980) or laboratory and field studies with

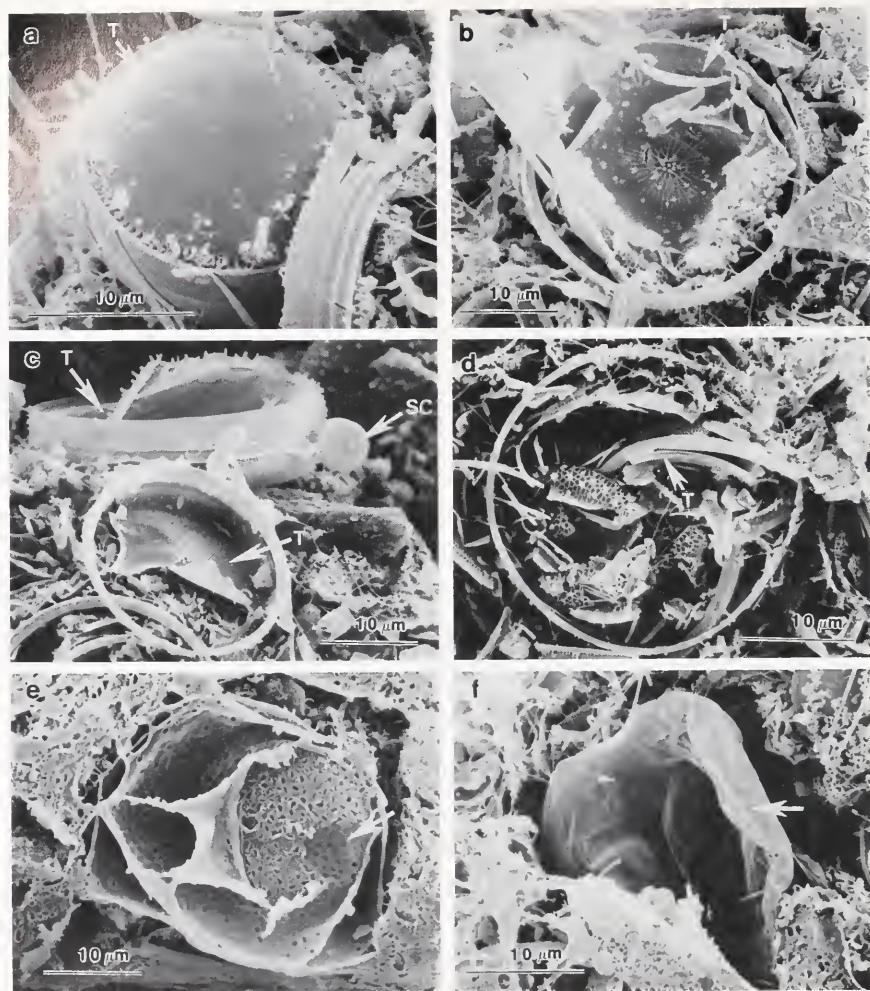


FIGURE 3. Contents of *Centropages velificatus* fecal pellets from the station on 24 November 1983 (0605). a. and b. *Thalassiosira* sp. cells (T). c. *Thalassiosira* sp. (T) and small centric (SC) diatom cells. d. *Thalassiosira* sp. fragments (T). e. *Prorocentrum compressum* cell (arrow). f. crustacean fragment (arrow).

congeners (Turner 1984a and references therein; Conley and Turner, 1985; Turner *et al.*, 1985).

The apparently frequent use of carnivory by *C. velificatus* is also suggested by quantitative grazing data from the northern Gulf of Mexico (Turner and Tester, 1986; Tester and Turner, 1986). Regression coefficients for grazing *versus* available phytoplankton gave poor correlation. Such uncoupling of grazing effort and phytoplankton abundance would be expected if *C. velificatus* feeds primarily as a carnivore, or selectively upon less-abundant phytoplankters.

Although the presence of primarily large phytoplankton cells in fecal pellets is suggestive of selective grazing on large particles, such a conclusion is not without ambiguities. For reasons detailed in Turner (1984c, p. 279) and (1984 d, p. 82), it is impossible to realistically make SEM analyses of fecal pellets quantitative. Further, it is not clear why *Prorocentrum compressum* cells were common in pellets but un-

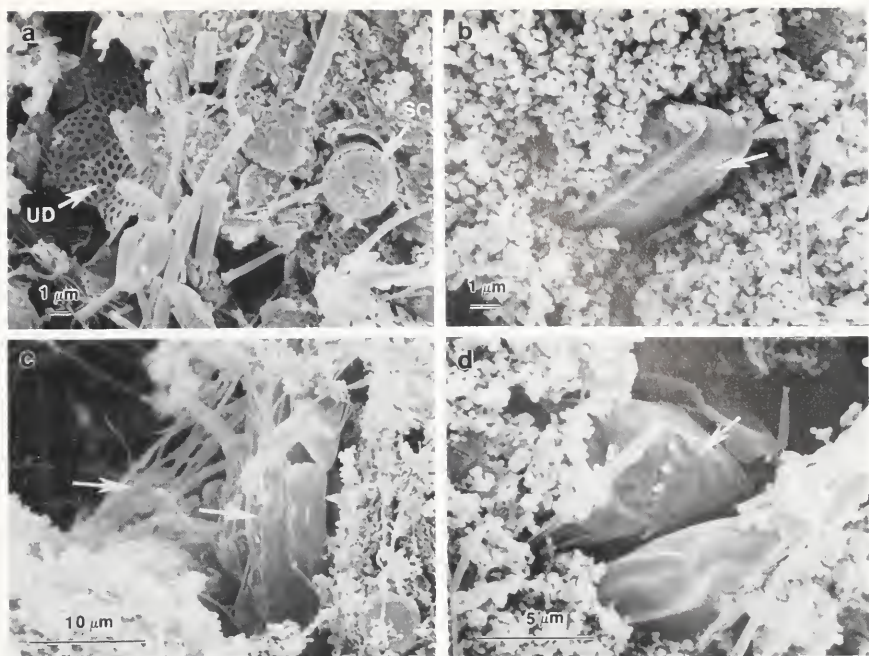


FIGURE 4. Contents of *Centropages velificatus* fecal pellets from the station on 22 November 1983. a. Fragments of an unidentified diatom (UD) and a small centric (SC) diatom. b. pennate diatom (arrow). c. and d. crustacean fragments (arrows).

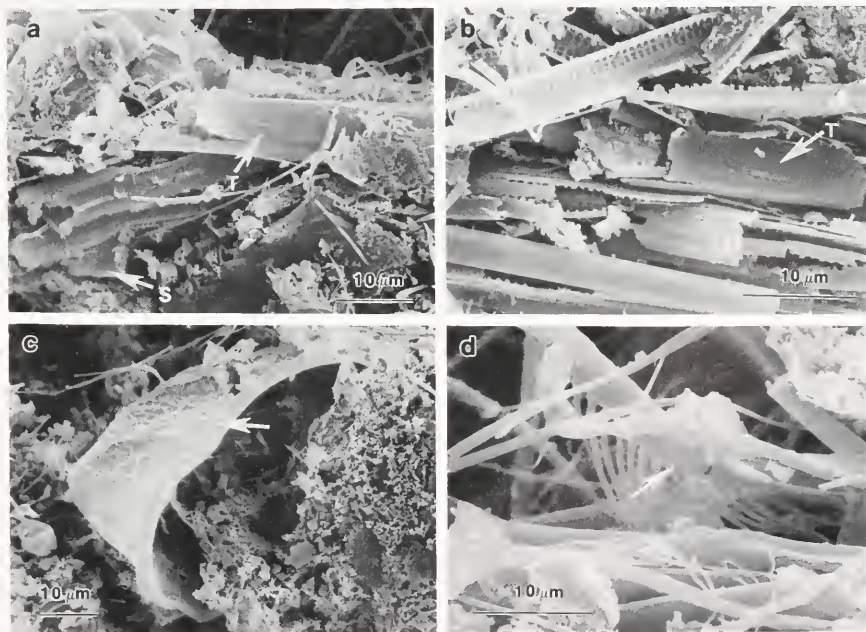


FIGURE 5. Contents of *Centropages velificatus* fecal pellets from the station on 27 November 1983. a. and b. *Thalassiothrix* sp. (T) and *Skeletonema costatum* (S) fragments. c. unidentified dinoflagellate fragment (arrow). d. crustacean remains (arrow).

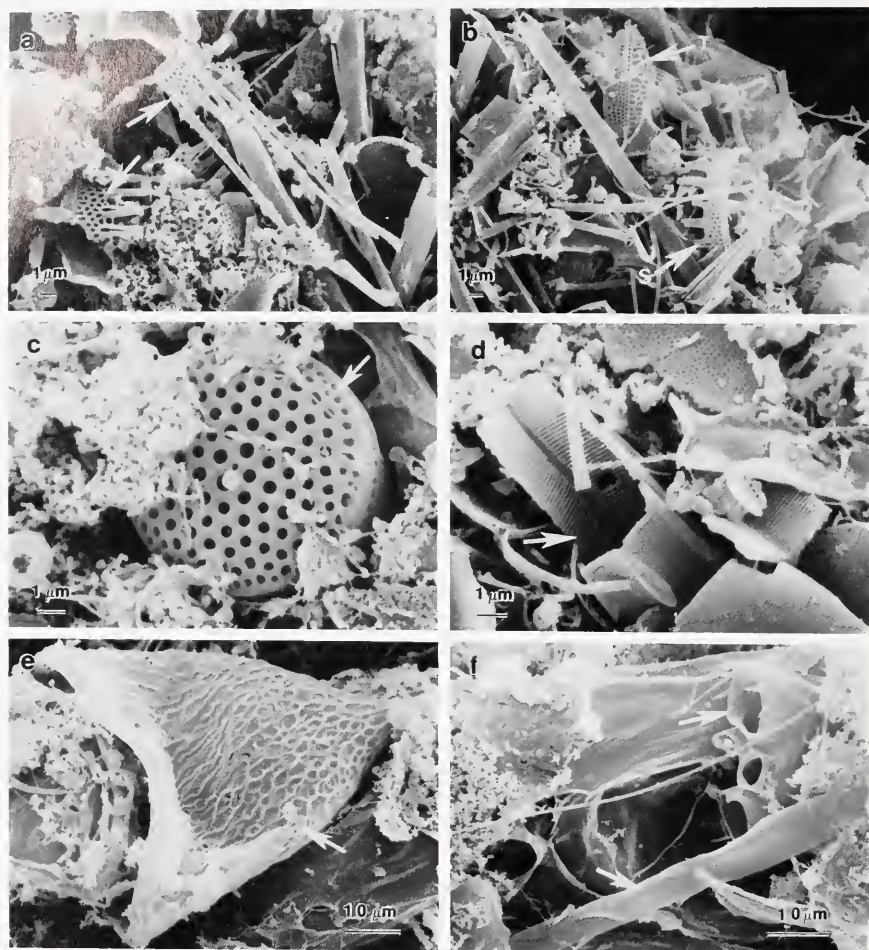


FIGURE 6. Contents of *Centropages velificatus* fecal pellets from the station on 30 November 1983. a. *Skeletonema costatum* fragments (arrows), b. *Skeletonema costatum* (S) and *Thalassiosira* sp. (T) fragments, c. intact centric diatom cell (arrow), d. *Thalassiothrix* sp. fragments (arrow), e. fragment of an unidentified dinoflagellate (arrow), f. crustacean remains (arrows).

common in the water (11% of total cells) on 15 December 1982, whereas the similarly large (33 μm diameter, or larger) diatom *Coscinodiscus radiatus* was not observed in these pellets even though it comprised 41% of available cells. In other cases, large *Thalassiosira* spp. cells were abundant in pellets, but since they were also abundant in the water, this pattern is more indicative of nonselective than selective feeding. Conversely, the disproportionately high abundances in pellets of elongate cells of *Thalassiothrix* sp. or *Skeletonema costatum* cells from elongate chains, when these taxa comprised only a small proportion of available cells, may indicate size selection on 27 and 30 November 1983. In short, there was no consistent pattern of ingestion of large *versus* small, solitary *versus* chain-forming, or diatom *versus* dinoflagellate cells. The production of fecal pellets containing primarily fine-grained silt particles reveals that small particles can also be ingested in large numbers. *Centropages velificatus* apparently employs a variety of mechanisms to capture food from a broad range

of particle sizes. Such varied feeding mechanisms have been demonstrated with high-speed cinematography for *Centropages typicus* (Cowles and Strickler, 1983) and other copepods (Price *et al.*, 1983).

The raptorial omnivory of *Centropages velificatus* evidenced by fecal pellet contents is atypical of many other copepod species studied with the same techniques. Mainly nonselective suspension feeding characterized *Eucalanus pileatus* (Turner, 1984b; Tester and Turner, 1986), *Paracalanus quasimodo* (Turner, 1984b), *Acartia tonsa* (Turner, 1984c; Tester and Turner, 1986), *Temora turbinata* and *T. stylifera* (Turner, 1984d), *Anomalocera ornata* (Turner, 1985), and *Undinula vulgaris* (Turner, 1986a). However, the frequent feeding of *C. velificatus* upon other crustaceans and larger, often less-abundant phytoplankters is similar to the patterns for *Labidocera aestiva* (Turner, 1984c) and several cyclopoids (Turner, 1986b). These results emphasize that when considering the trophic positions or impacts of "copepods" it is important to specify which ones.

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AN ANATOMICAL STUDY OF THE RETINA OF *NAUTILUS POMPILIUS*

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ABSTRACT

The receptor outer segments of *Nautilus* have several rhabdomeres of parallel microvilli occurring more or less equally at all orientations. The microvilli of adjacent receptors overlap substantially. The supporting cells of the retina possess both microvillous processes and small cilia. The receptor cells become disorganized towards their tips, although supporting cell processes can still be distinguished. There is no limiting membrane. Similarities to and differences from the retinas of adult and embryonic coleoid cephalopods are discussed.

The myeloid body of *Nautilus* is more developed than in other cephalopods, appearing in electronmicrographs as a complex of loops or circles. A series of dimpled plates stacked in register one above the other could generate most or all of these apparently complicated structures depending on the plane of section. Myeloid bodies occur both externally and internally to the nucleus, and occasionally within the outer segment.

At its margin the retina merges smoothly with the columnar epithelium lining the inside of the iris. The margin of the retina contains relatively more supporting cells than more central regions. Myeloid bodies first appear about 200 μm central to the point at which the retina starts to thicken.

INTRODUCTION

The Nautiloidea, a highly successful group of cephalopods which originated in the Cambrian period, are today only represented by a few species of *Nautilus* which are restricted to deep waters in tropical areas of the Pacific and possibly the Indian oceans. *Nautilus* differs in many respects from the more recent coleoid cephalopods and, although undoubtedly specialized in many ways for its deep-water habitat (Landman and Saunders, 1987), shows many characteristics that appear to have remained unchanged since Jurassic times. Therefore, its study is of special interest in that it may provide some insight into how the organization of the modern cephalopods arose.

The visual system is one of the features that may be primitive in *Nautilus*. The eye is structurally simple, consisting of a cup formed by an invagination of skin, communicating directly with the surrounding water by way of the open pupil. The outer and inner surfaces of the iris are lined with columnar epithelium bearing microvilli, which on the inside merges through a transitional zone with the retina itself; as in other cephalopods, the rhabdomeres of the outer segments of the retinal receptors are formed from modified microvilli (Messenger, 1981). The inner segments of the receptors contain nuclei and myeloid bodies [Merton's (1905) "phaosomes"]. The latter, which are probably concerned with visual pigment metabolism (Hara and

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Hara, 1972; Breneman *et al.*, 1986), are especially interesting because they are much more structurally complex than those found in the more recent coleoid cephalopods.

The structure of the eye has been the subject of a number of histological studies at both the light and the electron microscope levels (*e.g.*, Hensen, 1865; Griffin, 1900; Owen, 1932; Mugglin, 1937; Young, 1965; Barber and Wright, 1969; Muntz and Raj, 1984). Behavioral studies have also shown, as would be expected in an eye that has no lens but operates on the principle of a pin-hole camera, that both visual acuity and sensitivity are poor in comparison to equivalent lens-bearing eyes (Muntz and Raj, 1984; Muntz, 1987).

The present paper presents further information on various aspects of retinal structure: in particular on the interrelationship between the rhabdomeres of neighboring receptors and the supporting cells, the transitional zone between the epithelium lining the inside of the iris and the retina, and the structure of the supporting cell processes and the myeloid bodies.

MATERIALS AND METHODS

Specimens of *Nautilus* were trapped off the main reef at Suva, at depths between 165 and 600 m, and kept in glass aquaria in an air conditioned room at water temperatures between 7 and 13°C. The water was changed regularly, and the animals fed occasionally on prawns. Under these conditions they remained alive for several weeks, and one animal showed reliable positive phototactic behavior after more than 11 months in the laboratory. However histological preparations show some degeneration of the retina after a prolonged period in the laboratory, and the optomotor response also becomes less reliable (Muntz and Raj, 1984). Therefore the present paper reports results on animals used within 3 or 4 days of capture.

Small pieces of iris and retina were fixed in 2% glutaraldehyde made up in either Sörensen's phosphate buffer or filtered seawater. After 1.5 h fixation the tissue was washed in several changes of buffer or seawater as appropriate. The tissue was then post-fixed in 1% osmium tetroxide for 1 h. In some cases this post-fixation was done in Fiji and the tissue taken through to 70% ethanol before being brought back for further treatment. In most cases, however, the tissue was stored in fresh buffer or seawater and the further treatment, including post-fixation, carried out at Stirling or Monash University. Following post-fixation the tissue was either block stained in 2% uranyl acetate for 1 h or immediately dehydrated. Either Emix (EMscope, Ashford, England) or Spurr's resin (Spurr, 1969) were used to embed the tissue. Blocks were sectioned on either a Reichert OM3 Ultratome or an LKB "Ultratome III." Thin sections (2–4 μ m) were taken and stained with Toluidine Blue (pH 9.0) and examined under the light microscope. Ultrathin sections were stained with a saturated solution of uranyl acetate in 50% methanol and post-stained in lead citrate. The sections were examined using a JEOL JM100C(X) or an AEI Corinth 275 electron microscope.

In this paper the words inner or internally, when applied to the retina, mean towards the center of the animal, while outer or externally mean in the opposite direction, that is towards the light.

RESULTS

Outer segments and supporting cells

Figures 1a and b show electron micrographs of sagittal sections through the outer segments and supporting cells of the retina, confirming the structure described by Barber and Wright (1969). The tubules or microvilli that make up the rhabdomeres

can be seen leaving the outer segments, and where the plane of section cuts across the microvilli they appear, as in other cephalopods (*e.g.*, Cohen, 1973), to be closely packed. The length of the microvilli varies from 2 to 6 μm between different rhabdomeres, but within a given rhabdomere their length does not change appreciably with position. The rhabdomeres are separated from each other by the supporting cells which contain a number of parallel processes extending over the whole depth of the outer segment layer of the retina (300–500 μm) and which, from their appearance in both transverse and longitudinal section, are clearly microvillar in nature. Figure 1b shows the origin of the supporting cell processes at the cell body. Small primary cilia are also found in the supporting cells (Fig. 3). It is not clear if they also occur in the receptor cells.

A tangential section through the outer segments is shown in Figure 2. The rhabdomeres radiate between the outer segments of neighboring receptors, separated by the processes of the supporting cells. The microvilli from neighboring receptors interdigitate, individual microvilli passing most, or perhaps all, of the way from one outer segment to another. Supporting cells have on average 46 processes ($\text{SD} = 9$, $n = 30$): on three occasions the processes from 2 or 3 supporting cells have apparently run together resulting in groups of about 90 or 150 processes.

Figure 4 shows, at the light microscope level, a sagittal section through the edge of the retina and the transitional zone between the retina and the iris. It can be seen that over about the outer third of their length the structure of the receptors becomes disorganized. Figure 5 shows the appearance of the outer region, near the receptor tips, as seen under the electron microscope. Processes belonging to the supporting cells can be seen, but the structure of the receptors themselves is no longer clearly organized into receptor cell bodies and rhabdomeres containing parallel microvilli. There is no sign of a limiting membrane overlying the outer ends of the receptors such as occurs in the coleoid cephalopods (Zonana, 1961; Yamamoto *et al.*, 1965).

Transitional zone between iris and retina

Figure 4 shows that at the junction of the iris and the retina there is a smooth increase in the thickness of the outer layer of the retina due to the increasing length of the receptor outer segments or supporting cell processes. The myeloid bodies do not start to occur at the point at which the retina thickens, but considerably further in: thus in preparations from four animals the distance from the first myeloid body to the edge of the retina (as indicated by the arrows in Fig. 4) ranged from 130 μm to 240 μm (mean 193 μm).

The edge of the retina also contains relatively few receptor cells relative to supporting cells compared to more central regions (Fig. 6). It is likely that the outer layers of the retina take on their final form at the same point as the myeloid bodies appear.

Structure of the myeloid bodies

The appearance of the myeloid bodies under the transmission electron microscope varied widely from a simple appearance of a number of wavy membranous structures, to a complex of loops, whorls, and circles (Figs. 7, 8, 9). The myeloid body is usually located in the inner segment of the receptors, although displaced myeloid bodies lying in the outer segment occasionally are found. In electron micrographs of 52 receptor inner segments in which both the myeloid body and the nucleus could be seen, the myeloid body lay internally to the nucleus on 33 occasions and externally on 20 occasions. The fine membranous structure of the myeloid body is clearly shown in Figures 8 and 9.

DISCUSSION

In the coleoid cephalopods that have been studied to date the arrangement of the retina as seen in tangential section is very precise. The basic plan, first described by Schulze (1869), is that each receptor outer segment carries a rhabdomere on two opposite faces, and that the rhabdomeres from four receptors combine to make a square rhabdome (Young, 1962)—originally thought by analogy with the anthropods to be the retina's basic functional unit. As a result of this structure the microvilli of the rhabdomeres form two orthogonal sets, oriented in the vertical and horizontal planes, respectively. There are some exceptions, and individual receptors can on occasion contribute to up to eight rhabdomeres (Zonana, 1961; Cohen, 1973), but the underlying plan is clear. The rhabdomeres of adjacent receptors remain separate, without any overlap in their microvilli. In contrast, each receptor outer segment in *Nautilus* has several rhabdomeres occurring more or less equally at all angles, and with substantial or complete overlap between the microvilli of neighboring receptors. There is no obvious regular grouping of the rhabdomeres from neighboring receptors to form a structure analogous to the rhabdome.

The orthogonal arrangement of the rhabdomeres of coleoid cephalopods, and the parallel relationship of the microvilli within them, is commonly assumed to underlie the ability of cephalopods to discriminate the plane of polarization of light (e.g., Saidel *et al.*, 1983). The orthogonal arrangement does not occur in *Nautilus*, but the microvilli remain parallel to each other within any given rhabdome, and the angular separation between each rhabdome is still considerable even though less than 90°. Plane polarized light should thus still be able to affect the receptors of *Nautilus* differentially, but without any precise or predetermined pattern between the plane of polarization and the set of receptors that would be affected.

The interdigitation of the microvilli of neighboring receptors shown in Figure 2 is also visible in the electron micrographs published by Barber and Wright (1969), although not mentioned in that paper. Such interdigitation of microvilli is not unique to *Nautilus*, but occurs, for example, in some arachnids [e.g., *Lampona* (Gnaphosidae), Blest, 1985] and is an arrangement that might be expected to affect visual acuity. Thus the simplest hypothesis relating retinal structure to acuity is that two objects will be discriminable when their retinal images are far enough apart that at least one receptor remains unstimulated between them (Helmholtz, 1924). Since microvilli overlapping will increase the separation between stimuli necessary to achieve this, a loss of resolution at the retinal level will result. However, in *Nautilus* the pin-hole arrangement for image formation appears to be the limiting factor for visual resolution with the retinal mosaic considerably finer than is needed for the quality of the image that is formed (Muntz and Raj, 1984). The overlapping of the microvilli, even if this halved the effective fineness of the retinal mosaic, should not affect the animal's ability to resolve detail. However, the overlap could benefit sensitivity, important for an animal living at depth in the sea, if sensitivity is limited to some degree by random retinal events that are unrelated to the stimulus, such as the spontaneous breakdown of visual pigment molecules or the spontaneous release of transmitters at synapses (visual "noise"). Overlap will increase the number of receptors simultaneously activated by a given stimulus, whereas "noise" events will presumably be independent in time between the different receptors. Provided the central nervous system is able to detect the simultaneity of messages from the receptors, sensitivity should be improved.

The retina of *Nautilus* also differs from that of the coleoid cephalopods in that the supporting cells have small processes that are ciliary in nature, as well as the microvillous processes extending between the receptors. Ciliary structures have not hitherto

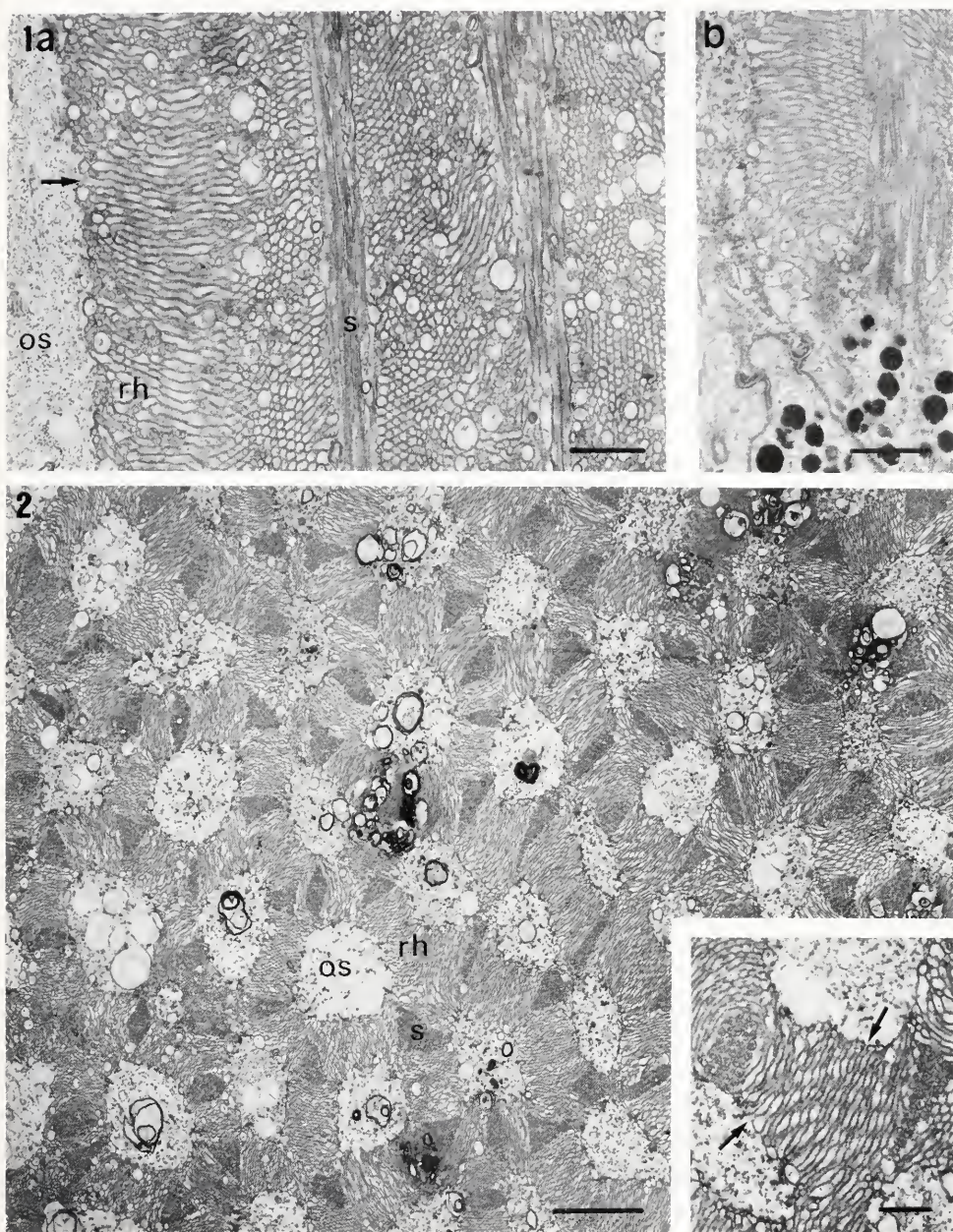


FIGURE 1. a. TEM of a sagittal section through the outer segments and supporting cells of the retina of *Nautilus pompilius*. os: outer segment, rh: rhabdomere, s: supporting cell. The arrow shows a microvillus opening into the outer segment. Fixative: 2% glutaraldehyde in seawater. Scale bar = 1 μ m. b. TEM of a sagittal section of the receptor and supporting cells showing the origin of the supporting cell processes and receptor microvilli. Fixative: 2% glutaraldehyde in seawater. Scale bar = 1 μ m.

FIGURE 2. TEM of a tangential section through outer segments and supporting cells of the retina. Labels as in Figure 1. Scale bar = 2 μ m. The insert shows the appearance at higher magnification. The arrows show interdigitating microvilli from neighboring receptors. Fixative: 2% glutaraldehyde in seawater. Scale bar = 1 μ m.

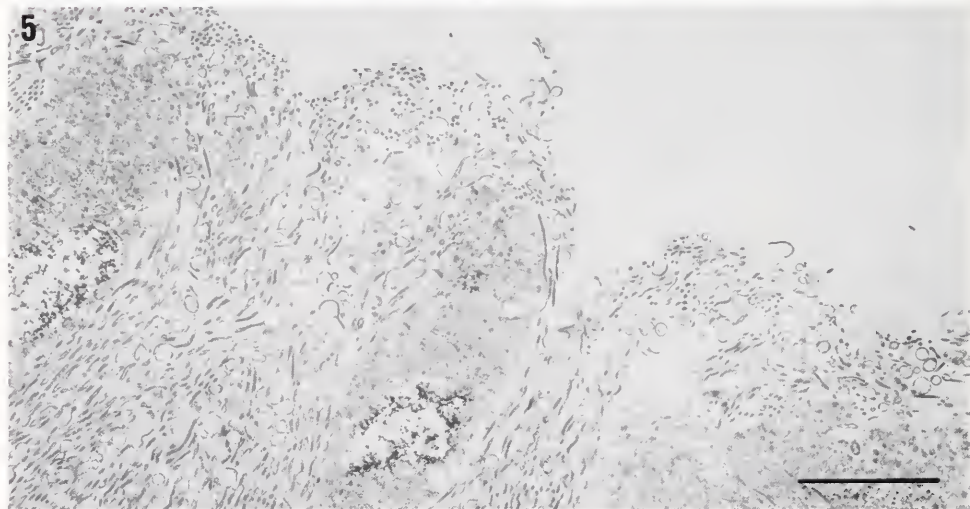
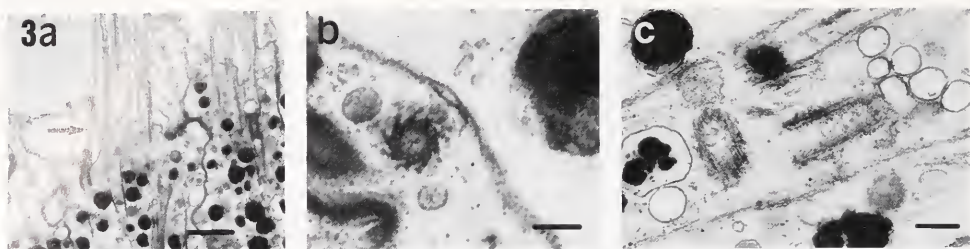


FIGURE 3. TEMs showing primary cilia in supporting cells and associated centriolar structures. Fixative: 2% glutaraldehyde in phosphate buffer. (a) Sagittal section of the retina. The cilium is indicated by the arrow. Scale bar = $1\ \mu\text{m}$. (b) Transverse section of the basal body of a cilium showing pinwheel structure. Scale bar = $0.2\ \mu\text{m}$. (c) Sagittal section of the retina showing diplosomal centrioles cut both longitudinally and obliquely. Scale bar = $0.2\ \mu\text{m}$.

FIGURE 4. LM of a sagittal section through the edge of the retina. The arrows show (1) the point at which the outer segments start to lengthen and (2) the first visible myeloid body. Fixative: 2% glutaraldehyde in seawater. Scale bar = $100\ \mu\text{m}$.

FIGURE 5. TEM of a sagittal section through outermost tips of the receptors. Supporting cell processes are still clearly visible. Fixative: 2% glutaraldehyde in phosphate buffer. Scale bar = $5\ \mu\text{m}$.

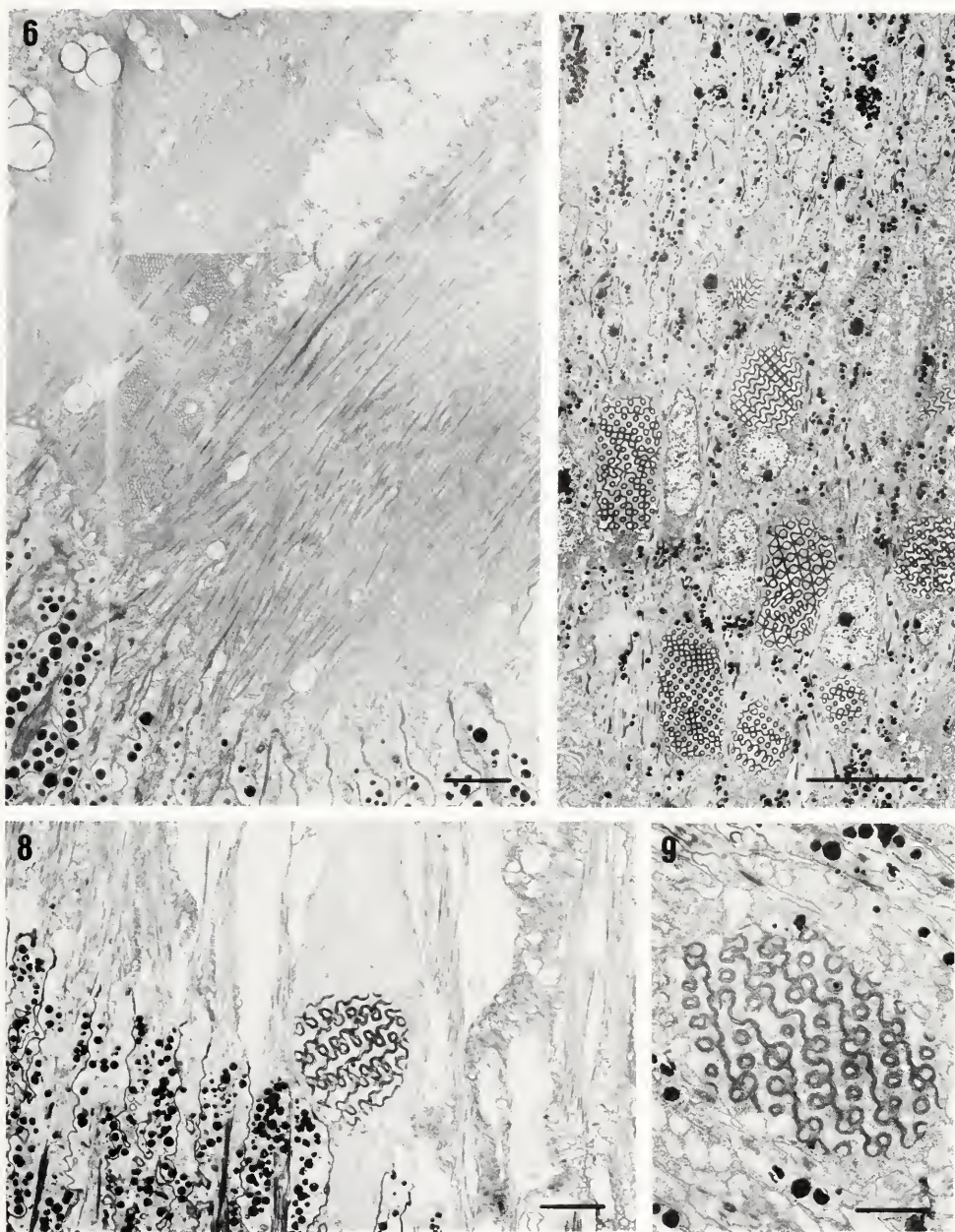


FIGURE 6. TEM of a sagittal section through the outer segments at the transitional zone at the edge of the retina. The iris is to the right. Fixative: 2% glutaraldehyde in seawater. Scale bar = 2 μ m.

FIGURE 7. TEM of sagittal section through the inner segments of retinal receptors showing the relative positions of nuclei and myeloid bodies. The bottom of the photograph is internal. Fixative: 2% glutaraldehyde in seawater. Scale bar = 10 μ m.

FIGURE 8. TEM of a sagittal section through the outer segments of the retina showing displaced myeloid body. Microtubules and supporting cell processes are clearly visible. Fixative: 2% glutaraldehyde in phosphate buffer. Scale bar = 1 μ m.

FIGURE 9. TEM of a sagittal section of a myeloid body showing membranous structure. Fixative: 2% glutaraldehyde in phosphate buffer. Scale bar = 2 μ m.

been reported in the retina of any adult cephalopod. The photosensitive organs of many animals have receptors of ciliary origin, or cilia, presumed not to be photosensitive, intermingled with the receptors (Vanfleteren, 1982). Many rhabdomeric receptors possess cilia in their embryonic or larval stages but not in the adult stage (Vanfleteren, 1982). Yamamoto (1985) reported that such structures are present in both the receptors and supporting cells of the embryos of the cuttlefish, *Sepiella japonica*, persisting longer in the supporting cells than in the receptors. The retina of the *S. japonica* embryo resembles that of *Nautilus* in a number of other respects. These include the fact that in early stages the nuclei of both supporting and receptor cells are external to the basal membrane, whereas in adults only the supporting cell nuclei remain external; that the microvilli of the receptor cells occur initially more or less equally in all directions, only achieving the regular rectilinear arrangement of adults at a relatively late stage of development; and that the supporting cells in embryos send long microvillous processes between the receptor cells over the whole length of their outer segments. They also possess cilia.

The myeloid bodies of *Nautilus*, which are found in the inner segments of the receptors, are much more complex than those found in the coleoid cephalopods. Barber and Wright (1969) state that they are usually found externally to the nucleus, while Merton's (1905) figures show them equally on either side of the nucleus. In our preparations they lay internally to the nucleus more often than externally, and they were also on occasion found displaced into the outer segment.

Myeloid bodies did not occur at the retinal margin, but only some 100–200 μm inwards after the point at which the retina outer layer starts to lengthen. The edge of the retina also contains relatively few rhabdomeres and many supporting cells. The presence of the myeloids may thus correlate with the presence of rhabdomeres.

The structure of the myeloid bodies has been described from light microscope studies as a stacked series of cubes, or a honeycomb structure (Merton, 1905), and from electron micrographs as a complex tubular array (Barber and Wright, 1969). Both Barber and Wright's electron microscope results and those shown in Figures 7, 8, and 9 of the present paper appear to confirm both the complexity of and on many occasions an apparently tubular structure. However, the photographs often give the impression of a series of stacked wavy plates, or of a structure where both forms of organization alternate or merge.

Despite the apparent complexity, there is a comparatively simple structure that can generate most or all of these different appearances depending on the plane of section, namely a series of dimpled plates stacked in register one above the other. Mathematically the surface of each individual plate can be approximated by the relationship $z = \cos x \cos y$ (Fig. 10). A section in the plane of the plates will clearly produce a pattern of circles, squared off to a greater or lesser extent depending on how close the section is to the point where $z = 0$, which could be interpreted as a tubular structure. Similarly, a section perpendicular to the plane of the plates will produce an appearance similar to a stacked series of wavy plates, the amplitude of the waves depending on how close the section is to the lines defined by $\cos x$ or $\cos y = 0$. Various intermediate patterns similar to those seen in the electron micrographs are generated when the section cuts the plate at an angle (see examples in Fig. 11). Of course these patterns will be repeated with a frequency which will depend on the spacing between the plates constituting the stack. The individual plates, or the whole stack, could also be curved, which would affect the appearance of the myeloid bodies.

The function of myeloid bodies remains uncertain. However the available evidence shows that in other cephalopods retinochrome is found in association with the myeloid bodies, and that the distribution of retinoids between the myeloid bodies

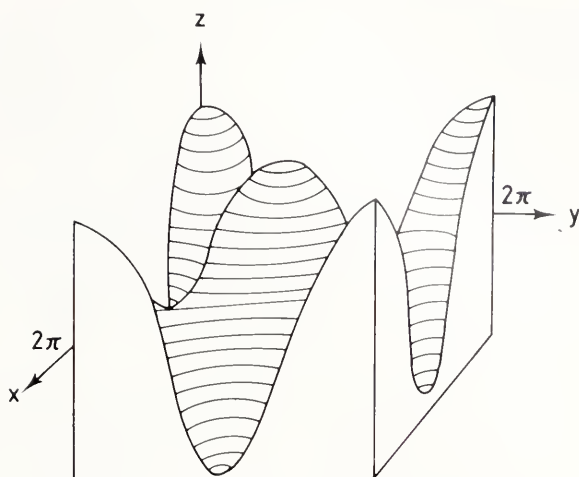


FIGURE 10A. Suggested structure of myeloid bodies. Appearance of a surface defined by $\cos x \cos y = z$.

and the outer segments of the receptors can be affected by the light regime under which the animals are kept (Hara and Hara, 1972; Breneman *et al.*, 1986). Thus the myeloid bodies are implicated in the visual pigment metabolism of cephalopods, most likely being concerned with pigment regeneration. Metabolic events often occur at the surfaces of membranes and thus depend on the membrane surface area. A stack of dimpled plates of the sort proposed represents a simple way of increasing the total surface area of membrane present in the myeloid body without increasing its gross dimensions. Why *Nautilus* should have a myeloid body that is so structurally complicated compared to other cephalopods remains unresolved.

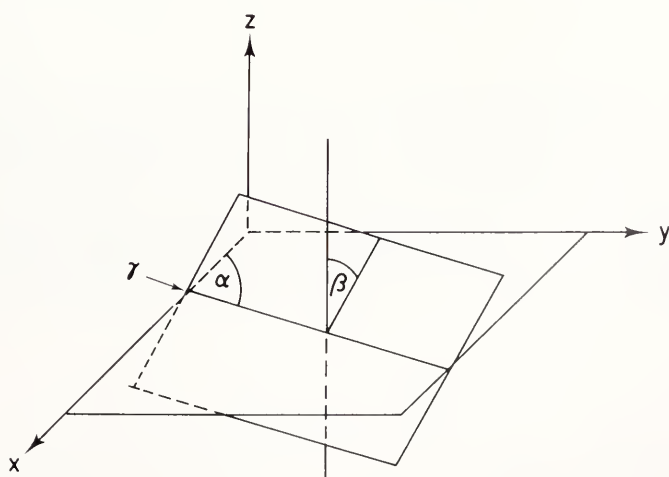


FIGURE 10B. Suggested structure of myeloid bodies. Any plane of section of the surface x, y can be defined by the two angles α and β and by the value of γ .

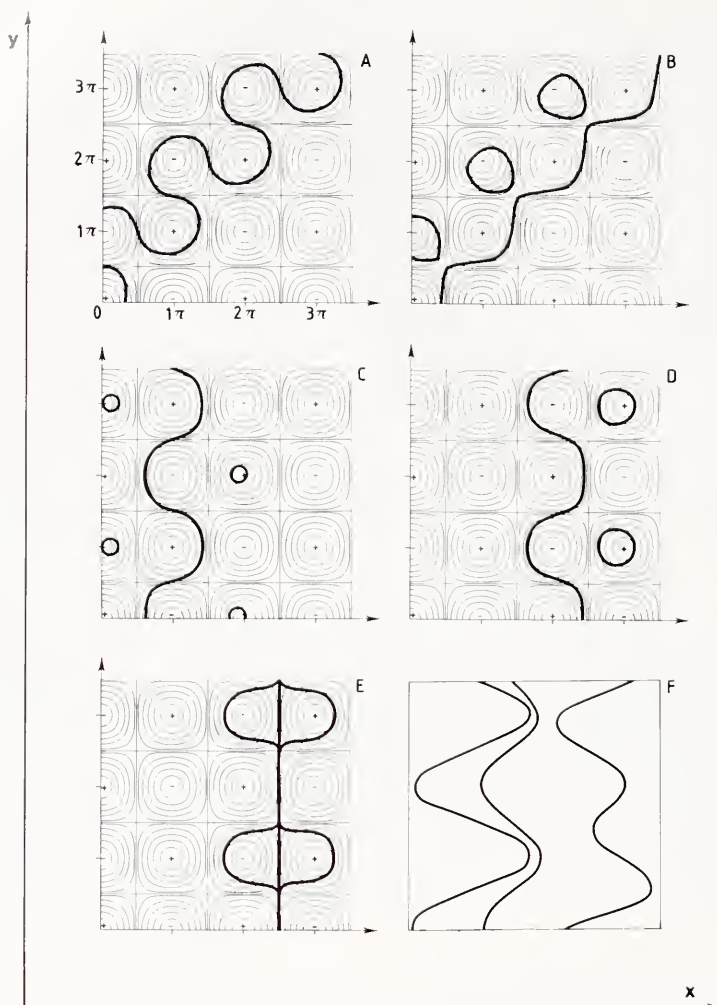


FIGURE 11. A to E, contour maps of the surface shown in Figure 10A, with the appearance of the cut surfaces that would be produced by sections cut in planes defined by different values of α and γ , β having a fixed value of π and z a maximum of 1 in all cases. The contour lines show heights and depths above and below the plane of the surface for values of 0, 0.2, 0.4, 0.6, and 0.8 of the maximum. A; $\alpha = 0.25\pi$, $\gamma = 1.5\pi$; B; $\alpha = 0.25\pi$, $\gamma = 0$; C; $\alpha = 0.5\pi$, $\gamma = \pi$; D; $\alpha = 0.5\pi$, $\gamma = 0.25\pi$; E; $\alpha = 0.5\pi$, $\gamma = 0.5\pi$. F, appearance of sections perpendicular to the plate (i.e. $\beta = 90^\circ$), for values of α of 0.5π and values of γ from left to right of 0.5π , 0.25π , and 1.75π respectively. Superimposed on a contour map such sections would appear as straight lines: they are shown here as they would be seen from a position perpendicular to the plane of section.

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SPECTRAL SENSITIVITY OF THE COMPOUND EYES IN THE PURPLE LAND CRAB *GECARCINUS LATERALIS* (FREMINVILLE)

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ABSTRACT

The spectral sensitivities ($S(\lambda)$) of dark-adapted compound eyes of the purple land crab *Gecarcinus lateralis* possess a broad maximum in the blue-green, 420–530 nm, when measured by electroretinographic (ERG) techniques. Selective adaptation experiments showed large changes in sensitivity but did not isolate different receptor types. A photopigment with maximal absorption at 487 nm was identified in the rhabdoms by microspectrophotometry. Besides the presence of a dominant green receptor system, the existence of a hump in the short wavelength region in $S(\lambda)$ suggests the presence of a blue-sensitive system as well. It is hypothesized that two photopigments (P487 and P440) in conjunction with screening pigment(s) mediate broad visual maximum in the blue-green in the purple land crab.

INTRODUCTION

One or two receptor systems have been described in the intertidal and subtidal crabs. Behavioral evidence for color discrimination exists for portunid crab *Carcinus* (von Buddenbrock and Friedrich, 1961) and for two *Uca* species (Hyatt, 1975). This can be accounted for by the presence of different receptor types produced either by different visual pigments (Wald, 1968) or by a single pigment in conjunction with screening pigments (Leggett, 1979). To date, microspectrophotometric (MSP) studies have revealed only a single visual pigment in crabs (e.g., spider crab *Libinia emarginata*: λ max 493 nm, Hays and Goldsmith, 1969; blue crab *Callinectes sapidus*: λ max 500 nm, Bruno and Goldsmith, 1974; green crab *Carcinus maenas*: λ max 505 nm, Bruno *et al.*, 1973, crab species. Cronin and Forward, 1987).

Electrophysiological studies have produced varying results regarding the types and numbers of photoreceptors in eyes of intertidal and subtidal crabs, even in the same species. Some workers using electroretinographic (ERG) or single unit recordings have located only a single receptor system. For example, Scott and Mote (1974) observed a single maximum at 510 nm in crabs inhabiting diverse habitats (*Callinectes sapidus*, *Sesarma reticulatum*, *Uca pugnator*, and *U. pugnans*). Wald (1968) reported that the subtidal *Libinia emarginata* had a single receptor type (λ max 490 nm), and Fernandez (1973) located only one system in the deep-water species *Pleuroncodes planipes* (λ max 523 nm). Similarly, Bruno *et al.* (1973) described a single 493 nm maximum in *Carcinus maenas*. However other workers have uncovered an additional, shorter wavelength receptor system in several of these species: *C. maenas* (Wald, 1968; Martin and Mote, 1982), *C. sapidus* (Martin and Mote, 1982), and *U. pugnator* (Hyatt, 1975). Thus it seems likely that crabs in general are dichromats.

The land crabs are decapod Crustacea which have made the transition from water to land. During their evolutionary adaptation to a new niche, a selection of a new set of physiological and behavioral mechanisms differing from their ancestors would be expected which could produce alterations in their visual systems. The visual pigments of several intertidal crab species have been described (Cronin and Forward, 1987), and some semiterrestrial crab species have been investigated electrophysiologically as described in the previous paragraph. Nevertheless, the spectral characteristics of vision are not known for the purple land crab. The objective here is to fill this gap in our present knowledge. The land crabs live in burrows in the sand or in the mangroves. Most of them forage at twilight or at night in a light limiting condition.

MATERIALS AND METHODS

Animals

The purple land crabs (4–6 cm carapace width) used in these experiments came from the West Coast of Florida and were kept in containers in the laboratory at room temperature (21–23°C) with a daily light cycle of 12 h light and 12 h dark.

Experimental preparation

Electrical recordings were taken from the corneal surface in live, intact preparations. The crabs were immobilized by bandaging heavily with gauze and then further secured with adhesive tape. The eye of the crab is retractable, so it was immobilized with dental cement to ensure that it remained easily accessible for experimentation. The animals were allowed to dark-adapt for one hour. All the experiments were conducted during the photophase at room temperature (21–23°C).

Electrical recordings

ERGs elicited by illumination of the eye were recorded by a glass pipette (tip diameter 5–10 μm) filled with physiological saline and inserted underneath the cornea. The reference electrode, also filled with saline, was inserted into a small hole bored in the shell of the animal. An Ag-AgCl wire made the connection between the electrodes and the grid of a high impedance preamplifier. The output of the preamplifier was fed to a DC coupled cathode ray oscilloscope (CRO) and a chart recorder.

Optical system

A two-channel optical system was utilized (details given in Lall *et al.*, 1982). One beam was for testing and other for chromatic adaptation. The test beam was obtained from a 150 watt xenon arc operated at 7.5 amps. with a regulated power supply. The light beam passed a high intensity grating monochromator. Quartz lenses collimated the test beam and focussed it on the entrance of a quartz light pipe (3×360 mm). The adapting beam was obtained from a 500 watt tungsten quartz iodide lamp, and was collimated with a quartz lens and superimposed on the test beam using a beam splitter. The quantum flux in both light beams was controlled by calibrated neutral density filters. Both beams were interrupted by Uniblitz photographic shutters which controlled the duration of the test flash and adaptation times.

Intensity calibration

The quantum flux of the test and the adaptation beam were determined by using a calibrated PIN-10UV Schottky barrier photodiode with a Model 210A amplifier

(United Detector Technology, Inc.). The photodiode was positioned directly at the end of the quartz light pipe in the same way the eye was positioned during experimentation and its output read on the CRO.

Experimental procedures

Light flashes of varying duration (0.1–0.4 s) at 25 nm or 20 nm steps from 340 to 680 nm were administered over 5 log units of intensity change. The ERGs elicited by these flashes were recorded. To maintain a constant steady dark-adapted state, the test flashes were delivered at least 30 s to 90 s apart depending upon the intensity of the test flash.

Spectral sensitivity curves

The amplitude of the ERG was used as an index of the sensitivity of the eye to the quantum flux and the wavelength composition of the photic stimulus. The spectral sensitivity functions were obtained by first determining the number of photons needed to elicit a criterion amplitude of the ERGs for different stimulus wavelength. A plot of $1/Q$ as a function of wavelength gave the spectral sensitivity function. Two variations of the criterion method were used for determining the spectral sensitivity curves. (a) ERGs were recorded at different levels of intensity for a stimulus wavelength and $V/\log I$ function was obtained. Similar functions were obtained for all the selected stimulus wavelengths across the spectrum. The reciprocal of the quanta needed to elicit a chosen criterion amplitude response across the stimulus wavelengths, as a function of wavelength, gave the spectral sensitivity curve. This is a lengthy procedure, and in most cases a shorter one was adopted. (b) One person observed the CRO screen while another adjusted the intensity of the test flash with neutral density filters at each wavelength until the observer signaled that a criterion response (50 μV or 200 μV) had been met.

Several chromatic adaptation experiments were conducted. Either Corning glass filters: blue (Cs5-60) and orange (Cs2-73) for broad-band irradiation, or Baird Atomic Interference filters for monochromatic (440 nm and 610 nm) intense light were placed in the path of the adapting beam of the stimulator and the eye was allowed to chromatically adapt continuously during experimentation. The test flashes for determining the spectral sensitivity were superimposed on the chromatic adaptation beam. The spectral sensitivity curves under chromatic adaptation were obtained by using the second criterion method described above.

Microspectrophotometry

A single-beam instrument described in Cronin (1984) was used. Animals were dark-adapted for several days, following which eyes were removed and ground in 2.5% glutaraldehyde in pH 7.5 MBL crustacean Ringer's (Cavanaugh, 1956). After 15 minutes of fixation at 0°C, the mixture of eye debris and photoreceptors (rhabdoms) was centrifuged, resuspended in pH 7.5 Ringer's, and maintained at 0°C. Individual rhabdoms were scanned as described in Cronin (1984). The rhodopsin absorption spectrum was determined by taking the difference between the absorption of a fully dark-adapted rhabdom and the absorption of the same rhabdom after a 5 minute photobleach with bright white light. Data from 13 rhabdoms were averaged and fit with a Dartnall (1953) nomogram as described in Cronin and Forward (1987).

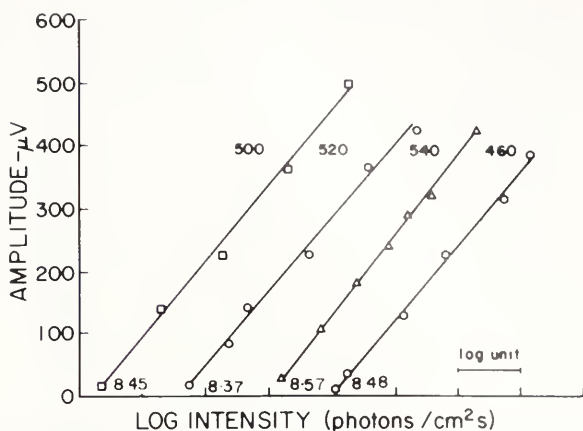


FIGURE 1. $V/\log I$ curves for a dark-adapted compound eyes in *Gecarcinus lateralis*. The number at the bottom of the curve is the log photons for the lowest response. Note that the curves for different wavelengths are similar in slope.

RESULTS

Electroretinograms (ERGs)

The ERGs were recorded from the corneal surface of the compound eyes after an initial latency period (20–60 ms) from the onset of illumination. The response was an “on” negative potential consisting of an initial phasic component followed by a maintained or plateau component which lasted for the total duration of the illumination. At low levels of illumination only the plateau component was recorded. The phasic component appeared at the intermediate levels of the illumination and increased sharply with bright illumination. The “on” negative ERGs in the land crab were similar to the ones recorded from the compound eyes of many arthropods which have scotopic eyes (*e.g.*, horseshoe crab; Chapman and Lall, 1967; Crustacea; Wald, 1968). The response waveform of the ERGs elicited by the stimuli of different wavelengths tended to be similar.

Intensity-response ($V/\log I$) functions

Figure 1 shows the amplitude of the phasic component of the ERG plotted as a function of log intensity of the stimuli of different wavelengths and intensities. The slopes of these $V/\log I$ functions for the phasic components did not vary with stimulus wavelength. These $V/\log I$ curves were used for: (a) determining the spectral sensitivities in Figure 3 (only the DA curve) and (b) determining whether there were any wavelength-dependent changes in the slope of the $V/\log I$ functions. Systematic changes in the slopes of the response curves for different wavelengths have been taken as evidence for the presence of different receptor types as in the median ocelli of *Limulus* (Chapman and Lall, 1967) and in the compound eyes of whirligig beetle (Bennett, 1967) and wolf spider (DeVoe *et al.*, 1969). The $V/\log I$ curves extended over 4 to 5 log units of intensity change, which indicated that the photoreceptor could function over 10,000 to 100,000 fold change in stimulus intensity.

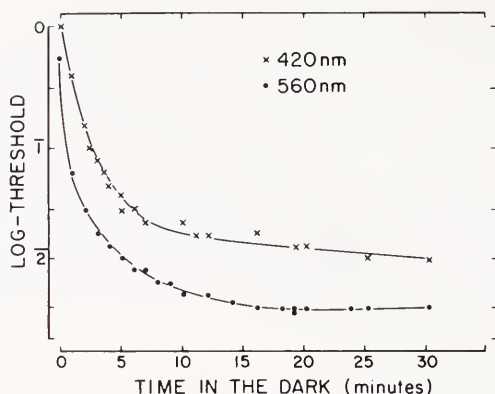


FIGURE 2. Dark-adaptation of the purple land crab compound eye measured at two wavelengths. The curves tended to be hyperbolic initially and follow a similar time course.

Dark-adaptation

Figure 2 shows the time course of dark-adaptation measured at two wavelengths (560 nm and 420 nm). The eye was light adapted for 1 minute with white light, and then the responses during dark-adaptation were tested alternately at 560 nm and 420 nm. Initially the threshold decreased hyperbolically, and then after about 20 minutes the threshold decrease was linear. These two curves are parallel, showing that the eye maintains constant relative sensitivities to the two wavelengths throughout dark adaptation.

Spectral sensitivity

Figure 3 shows the $S(\lambda)$ functions under dark- and chromatic adaptation conditions in *G. lateralis*. The dark-adapted $S(\lambda)$ curves showed a very broad sensitivity in the blue-green (440–520 nm) region of the spectrum. In two animals ($\blacktriangle$, Fig. 3), the sensitivity in the blue (430–460 nm) region was pronounced. Under chromatic adaptation conditions with both Corning glass filters and narrow band interference filters, the $S(\lambda)$ curves exhibited a decrease in sensitivity of about 1.5 to 2.5 log units, but a distinctive and pronounced selective effect with differential suppression of different parts of the spectrum was not observed. The chromatic adaptation curves tended to be broad and rather flat across the spectrum (compare curves a, b, and d for different chromatic adaptation conditions in Fig. 3), except for a small hump in the blue under red selective adaptation light. However, it should be noted that the ERG is a gross response from the whole eye, and chromatic adaptation experiments may poorly separate different receptor types even when the receptors are as far apart in the spectrum as near-UV and green (e.g., *Dineutes*; Bennett, 1978; *Photuris versicolor*; Lall, 1981). This difficulty in receptor isolation is further compounded when the receptors are as adjacent in the spectrum as blue *versus* green, and when the number of blue receptors is only a very small fraction of the green as in the blue crab *Callinectes* (Martin and Mote, 1982).

Microspectrophotometry (MSP)

The absorption spectrum of the visual pigment found in the rhabdoms of *Gecarcinus lateralis* is shown in Figure 4 and represents an average curve of the difference

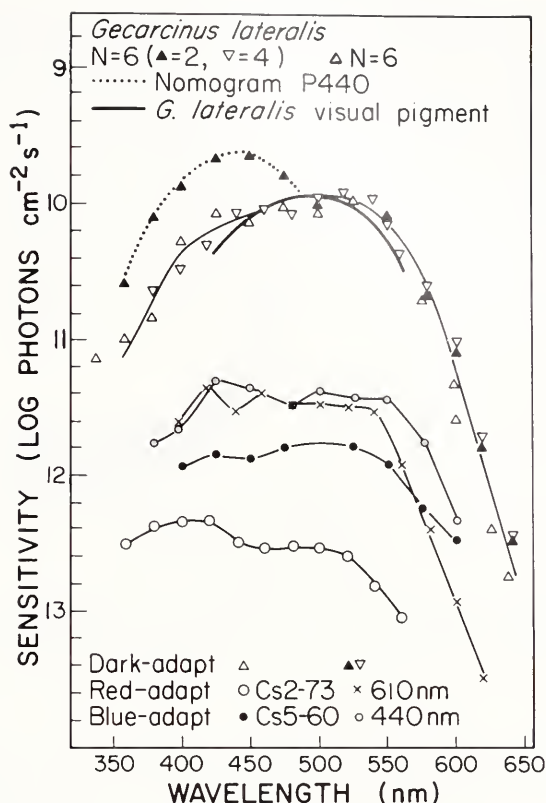


FIGURE 3. Spectral sensitivity of the purple land crab compound eyes under dark- and chromatic adaptation conditions. Nomogram (Ebbrey and Honig, 1977) for P440(.....) and *G. lateralis* visual pigment (—) from Figure 4 are superimposed for the data.

spectra between bleaches and dark-adapted preparations of 13 individual photoreceptors. The curve possesses a peak in the blue-green. The Dartnall (1953) nomogram curve for P487 nm closely matches this difference spectrum curve (Fig. 4).

DISCUSSION

The purple land crab *Gecarcinus lateralis* possesses a primary blue-green sensitive receptor system. This is supported by the following observations: (a) similarity of the ERG waveforms as a function of wavelength, (b) univariance of $V/\log I$ slopes as a function of wavelength (Fig. 1), (c) broad dark-adaptation curves with maximum in the blue-green (Fig. 3), (d) a lack of isolation of either blue or green receptor system under conditions of chromatic adaptation (Fig. 3), and (e) the presence of a blue-green absorbing (λ max 487 nm) photopigment in the rhabdoms (Fig. 4). However this does not rule out the possibility of a second photoreceptor system, since it is possible that the contribution of a receptor type consisting of only few ommatidia can be masked by the dominant receptor system. In the retina of the swimming blue crab *Callinectes sapidus*, green-sensitive cells (λ max 508 nm) were dominant, while only a few cells restricted to the ventral border region were blue-sensitive (λ max 440

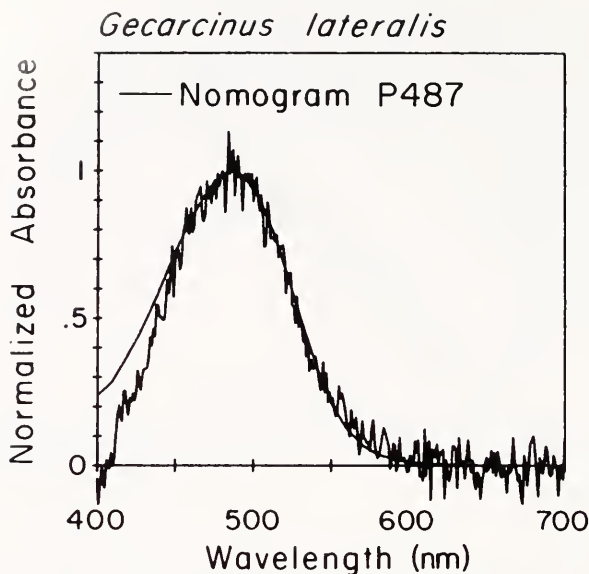


FIGURE 4. Average absorption spectrum of the rhodopsin in 13 rhabdoms determined microspectrophotometrically by taking the difference between spectra obtained from rhabdoms when dark-adapted and again when photobleached with white light. The solid line represents a Dartnall's nomogram curve for P487.

nm, Martin and Mote, 1982). Consequently, earlier studies using both single cell (Scott and Mote, 1974) and ERG (Goldsmith and Fernandez, 1968) techniques did not uncover the blue-sensitive receptor system. In our data, the presence of high sensitivity in the blue in a few recordings strongly suggests that a similar blue-sensitive receptor system (P440) exists in the purple land crab (Fig. 3).

It should be noted that the peak of the $S(\lambda)$ function in *G. lateralis* is difficult to establish in our ERG data (Fig. 3). The maximal sensitivity in the blue-green is much broader than the absorption spectrum of the most prevalent visual pigment, P487 (Fig. 4), presumably responsible for the sensitivity in the green (Fig. 3). The presence of screening pigments have been implicated in modifying the visual spectral sensitivity mediated by the visual pigment(s). This modification in some Crustacea has led to a broadening of the $S(\lambda)$ functions in the green (*i.e.*, *Leptograpsus variegatus*, Stowe, 1980; *Carcinus maenas*, Wald, 1968, Bruno *et al.*, 1973). While in other species a shifting of the lambda maximum of the $S(\lambda)$ functions to longer wavelengths occurs. For instance, in the lobster the peak of the ERG $S(\lambda)$ function is at 525 nm (Kennedy and Bruno, 1961), whereas the peak of the difference spectrum for bleaching of lobster visual pigment is at 500 nm (Wald and Hubbard, 1957; Bruno *et al.*, 1977). Similarly, in the crayfish the species visual pigment λ max appears to be at 530–535 nm (Goldsmith, 1978a) whereas the peak of the ERG $S(\lambda)$ function is at 565–570 nm (Kennedy and Bruno, 1961; Goldsmith and Fernandez, 1968; Wald, 1968), a dramatic shift of about 35 nm towards the red. These bathochromic shifts in the peaks of the $S(\lambda)$ functions from the peaks of the species rhodopsins have been attributed to the presence of distal screening pigment granules in some Crustacea (crayfish: Goldsmith, 1978b; crab *L. variegatus*: Stowe, 1980). It is quite conceivable that P487 overlaid by screening pigment(s) could cause a broadening of the $S(\lambda)$ functions in blue-green in the purple land crab.

The purple land crab *G. lateralis* generally inhabit tropical coastal hammocks (Bliss *et al.*, 1978). For protection the land crab burrows into the sand; it never wanders too far away from the safety of its burrow. The crab is active under environmental conditions of low ambient light, temperatures from about 18.5°C to 30°C, and high humidity. Unlike the giant crab, *Cardisoma guanhumi*, *G. lateralis* is not primarily nocturnal and has been observed to be active in the subdued illumination under heavy vegetation during the day. For effective functioning in a such an environment, a medium sensitivity, low threshold receptor with maximal sensitivity in the green would be ideal for the purple land crab. The presence of a blue receptor would enable the crab to discriminate open space from closed space. Our data suggest that indeed *G. lateralis* possesses its highest sensitivity in the blue-green region of the spectrum.

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CELL VOLUME REGULATION BY MOLLUSCAN ERYTHROCYTES DURING HYPOOSMOTIC STRESS: Ca^{2+} EFFECTS ON IONIC AND ORGANIC OSMOLYTE EFFLUXES

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ABSTRACT

The role of Ca^{2+} in volume regulation remains obscure. Before it can be investigated, however, the time courses of osmolyte and cell volume regulation and the effect of Ca^{2+} must be simultaneously specified in a suitable cell type. We have tested the red blood cells of *Noetia ponderosa* in that context. Our results show that the regulation of cell volume of the erythrocytes following hypoosmotic stress has two components. The first is an efflux of intracellular K^+ and Cl^- (but not Na^+) that begins immediately with the onset of hypoosmotic exposure. The second component, an efflux of taurine, follows the first, but only after many minutes. In addition, clam erythrocyte volume regulation is dependent on external $[\text{Ca}^{2+}]$. Volume recovery is potentiated in hypoosmotic media containing elevated Ca^{2+} levels. Taurine efflux from clam erythrocytes in hypoosmotic conditions is reduced in Ca^{2+} -free media and potentiated in high Ca^{2+} media. In contrast, the effluxes of K^+ and Cl^- are not sensitive to extracellular Ca^{2+} levels in either isosmotic or hypoosmotic media. Thus, the effluxes of ionic and organic osmolytes from these cells are controlled by mechanisms that differ in response time and Ca^{2+} sensitivity. These results suggest that the clam cells have an unexceptional volume regulatory mechanism and should therefore make an excellent model with which to study the role of Ca^{2+} in that process.

INTRODUCTION

Cell volume in response to hypoosmotic stress is controlled by regulation of the amount of intracellular osmolytes. Cell volume increases rapidly when water enters in response to hypoosmotic exposure, but then decreases as an efflux of intracellular osmolytes removes osmotically obligated water.

The cells of marine invertebrates use intracellular free amino acids (FAA, for review see Pierce, 1982) or other small nitrogenous compounds (proline betaine, Pierce *et al.*, 1984; glycine betaine, Warren and Pierce, 1982) as osmolytes. In some invertebrate cell types, an intracellular inorganic component to cell volume regulation has been found in addition to the organic osmolytes (*Limulus* myocardium, Warren and Pierce, 1982; *Glycera* coelomocytes, Costa and Pierce, 1983; *Noetia* red blood cells, Smith and Pierce, 1983; *Cancer* leg muscle, Moran and Pierce, 1984). The ions (Na^+ , or K^+ , and Cl^-) leave the cell as soon as the hypoosmotic stress begins, and the organic efflux follows at a slower rate.

In addition to the above characteristics, cell volume regulation in response to a hypoosmotic stress seems to be affected by external Ca^{2+} concentrations (for a review see Pierce and Amende, 1981; Pierce, 1982). Generally, when Ca^{2+} is reduced or

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Abbreviations: FAA, free amino acids; ASW, artificial seawater; EGTA, ethyleneglycol-bis-(β -amino ethyl ether) N,N'-tetraacetic acid.

omitted from the medium, cell volume regulation is either blocked or reduced. Free $[Ca^{2+}]_i$ is too low to exert a substantial osmotic effect. Thus, the role played by Ca^{2+} in the volume recovery must be either to activate or to regulate the osmolyte efflux mechanism. Since understanding that regulatory mechanism is a central issue in cell volume regulation, we have begun to investigate the Ca^{2+} effects more closely.

Unfortunately, the characteristics of the volume regulatory mechanism have not been examined in sufficient detail in any single cell type to serve as a point of departure. Therefore we have selected a single cell type, the red blood cell of the bivalve, *Noetia ponderosa*, and have described the entire process of osmolyte regulation during the volume response to a hypoosmotic stress on this system. In addition, we have determined the effect of $[Ca^{2+}]_0$ on each step of the process. Our results provide the first simultaneous chronology of all the events (ion, amino acid, and volume changes) that constitute cell volume recovery from a hypoosmotic stress, including the effects of Ca^{2+} on that chronology. In addition, the similarity of our results to those from other cells indicates that the *Noetia* cells are a useful model with which to study the effects of Ca^{2+} further. A preliminary report of this study has been published earlier (Smith and Pierce, 1983).

MATERIALS AND METHODS

Preparation of erythrocytes

N. ponderosa were collected by commercial dredge and maintained as described by Amende and Pierce (1980a).

Blood was collected from the clams at room temperature by forcing the valves open and slashing the mantle. The blood was diluted with artificial seawater (ASW, 935 mosm) made according to Amende and Pierce (1980) and buffered to pH 7.4 with 5 mM MOPS. The blood was then filtered through polyester wool. The erythrocytes were centrifuged twice at $500 \times g$; the supernatants were discarded and the cells were resuspended in ASW between the spins. After the second centrifugation, the cells were resuspended in ASW and centrifuged at $3000 \times g$, and the top layer of reproductive and amoebocytic cells was removed by aspiration. The erythrocyte pellet was washed twice more and the erythrocytes were then resuspended in approximately 1 ml of ASW for further use.

Measurement of cell volume regulation

Cell volumes of *Noetia* erythrocytes were measured in isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW, or in iso- or hypoosmotic media containing increased Ca^{2+} or no Ca^{2+} (Table I). Hypoosmotic ASW was made by diluting isosmotic ASW with glass distilled water. MOPS was always kept at 5 mM, and the pH at 7.4. The osmotic concentrations of the solutions containing 150 mM Ca^{2+} were matched to the control solutions by reducing the NaCl content. The high Ca^{2+} isosmotic solution was made fresh just before use as follows: first, the chloride salts of Na^+ , K^+ , and Mg^{2+} were dissolved in distilled water in the concentrations indicated in Table I. $CaCl_2$ was then added to the mixture to produce a final concentration of 150 mM. Finally, predissolved solutions of Mg_2SO_4 and $NaHCO_3$ were added very slowly with stirring to produce the concentrations of the ions listed in Table I.

NaCl content was increased to account for the osmotic deficit produced by Ca^{2+} removal in the Ca^{2+} -free ASW. This last solution also contained 1 mM EGTA. The osmotic concentrations of all solutions were determined before use with a vapor pressure osmometer (Wescor, model 5100C).

Approximately 4.0×10^6 washed cells were suspended in each experimental me-

TABLE I

Ionic composition (in mmoles/liter) of isosmotic artificial seawaters used as experimental media

Ion	Control	Ca ²⁺ -Free	High Ca ²⁺
NaCl	389.0	400.1	249.6
MgCl ₂	24.4	24.4	24.4
CaCl ₂	9.7	0	150.1
KCl	9.7	9.7	9.7
MgSO ₄	27.0	27.0	27.0
NaHCO ₃	2.3	2.3	2.3
EGTA	—	1.0	—
MOPS	5.0	5.0	5.0

dium and the distribution of cell volumes in a 10,000 cell aliquot was measured after 5, 10, 20, 60, and 120 min of exposure to the media using an electronic cell sizer (Coulter Counter Model ZB) and Coulter Channelyzer (Coulter Electronics Inc., Hialeah, FL) interfaced with an Apple II+ computer. The time course of erythrocyte volume change was measured in both high Ca²⁺ hypoosmotic and Ca²⁺-free hypoosmotic ASWs following a 30 min preincubation of the cells in 935 mosm ASW containing high Ca²⁺ and no Ca²⁺, respectively.

Mean cell volumes from a given experiment were expressed as a percent of the mean cell volume at zero time. The differences in the percent initial cell volume in experimental media at all sampling times were tested using analysis of variance and the Student-Newman Keuls multiple range test (Steele and Torrie, 1960). $P \leq 0.05$ was considered significant.

Taurine efflux measurements

The amino acid efflux from *Noetia* erythrocytes exposed to hypoosmotic media consists primarily of taurine (Amende and Pierce, 1980). In addition, our preliminary experiments showed that when a treatment altered the amino acid efflux, the release of each amino acid was changed by the same proportion; the effluxes of the amino acids were not altered differentially. In particular, taurine always accounted for 60–70% of the total FAA efflux regardless of the magnitude of the FAA efflux. Therefore, we used taurine to represent amino acid efflux.

Clam erythrocytes were removed from the animal and prepared as described above. The cells were then placed into one of the experimental media. At appropriate intervals, aliquots containing $4\text{--}7 \times 10^6$ cells were removed from the experimental cell suspension and centrifuged at $5000 \times g$ for 10 min. The taurine content of the supernatant was then determined as follows. An equal volume of 80% ethanol was added to the supernatant and the solution was placed into a boiling water bath for 20 min. Precipitated protein was removed by centrifugation at $25,000 \times g$ for 20 min. The supernatant was then lyophilized, the residue taken up in 0.2 N lithium citrate buffer, pH 2.2, and the taurine content measured with an amino acid analyzer (JEOL JLC-6AH). The cell number in three 15–20 μl aliquots from each cell suspension was counted three times with the Coulter Counter and averaged to express the taurine levels in terms of cell number.

Significant differences in taurine efflux were determined by analysis of variance and the Student-Newman Kuels multiple range test.

Intracellular K⁺, Na⁺, and Cl⁻

Erythrocytes were isolated as described above, and $10\text{--}20 \times 10^6$ cells were suspended in isosmotic media. Six 0.2 ml aliquots (approximately $1\text{--}8 \times 10^6$ cells) were then removed for measurement of intracellular K⁺, Na⁺, or Cl⁻ at zero time. The rest of the isosmotic suspension was divided in half and both halves centrifuged at $3000 \times g$ for 5 min. The supernatants were aspirated and the cells resuspended in either isosmotic or hypoosmotic media and sampled at intervals. At each sampling time, triplicate 0.2 ml aliquots (approximately $1\text{--}8 \times 10^6$ cells) were removed from the experimental cell suspension for the measurement of intracellular K⁺, Na⁺, or Cl⁻.

Intracellular K⁺ was measured by the method of Costa and Pierce (1983). The 0.2 ml aliquot of cell suspension was layered onto 0.1 ml of silicone oil (Wm. F. Nye, Inc., New Bedford, MA 02742, USA) which had been layered over 0.1 ml of 25% glycerol. This preparation was then centrifuged at $3000 \times g$ for 3 min (Beckman Microfuge II) which pelleted the cells through the oil into the glycerol where they lysed. The experimental medium remained on top of the oil. After the medium and oil layers were aspirated, the glycerol phase containing lysed erythrocytes was prepared for K⁺ measurement using atomic absorption spectroscopy (Perkin-Elmer model 560).

The amount of medium carried with the cells as they moved through the oil into the glycerol was determined for parallel replicates of cell suspension by the method of Freel *et al.* (1973) using ¹⁴C-PEG as an extracellular space marker. The K⁺ content of the trapped medium (usually about 1% of the total cell pellet K⁺) was subtracted from the K⁺ content of the glycerinated cell pellet to yield intracellular K⁺.

Intracellular Na⁺ was measured using the same technique described above for K⁺, except that the trapped volume was measured directly for each sample instead of for parallel replicates. Since ASW Na⁺ concentrations are high, the Na⁺ level of the trapped medium could account for up to 60% of total cell pellet Na⁺. After the cells had been sampled as described above, the medium and oil layers were aspirated, and an additional 0.1 ml of 25% glycerol was added to the cell pellet. This glycerol suspension was homogenized, and 0.05 ml was removed for trapped volume estimation. The remaining 0.15 ml was used to measure cellular Na⁺ content by atomic absorption spectroscopy.

The technique for determining intracellular Cl⁻ was identical to that used for Na⁺, except that Cl⁻ was measured by amperometric titration (Chloridometer, Buchler-Cotlove).

Statistical differences in intracellular K⁺, Na⁺, and Cl⁻ content were determined using analysis of variance and the Student-Newman Kuels multiple range test.

RESULTS

Noetia erythrocytes swell rapidly in hypoosmotic ASW, but within 10 min ($P \leq 0.05$) after hypoosmotic exposure they begin to recover towards their original volume (Fig. 1). Although volume recovery continues for at least two hours after hypoosmotic exposure, very close to half of the volume decrease happens within 20 min of hypoosmotic exposure.

Taurine efflux from *Noetia* erythrocytes in hypoosmotic media increases significantly during the volume regulatory period. However, most of the taurine efflux occurs between 10 and 60 min of hypoosmotic exposure (Fig. 2).

There is no significant difference between the Na⁺ content of isosmotic and hypoosmotic cells over the duration of the two hour time course (Fig. 3a). However, intracellular K⁺ decreases by about 15%, from 98 to 85 nmoles/ 10^6 cells within 10 min

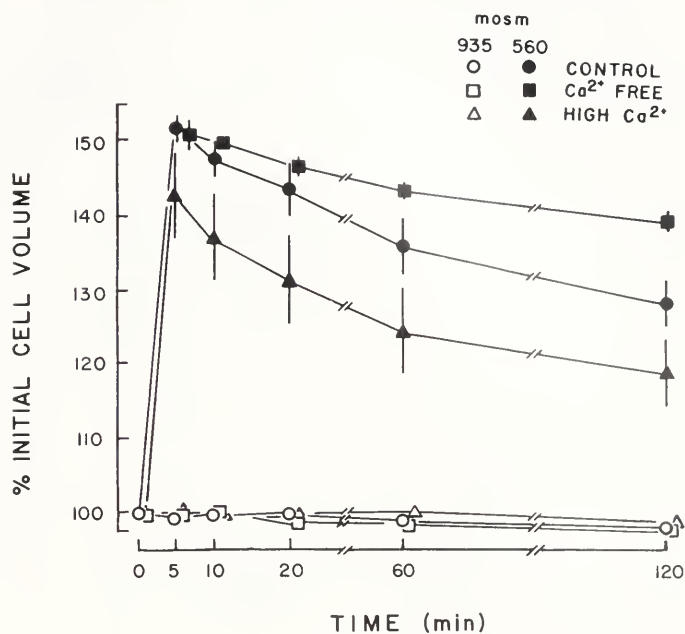


FIGURE 1. Volume changes of *Noctia ponderosa* erythrocytes exposed to isosmotic (935 mosm) or hypoosmotic (560 mosm) ASW of varying Ca^{2+} concentration. Vertical bars are ± 1 standard error of the mean (S.E.M.).

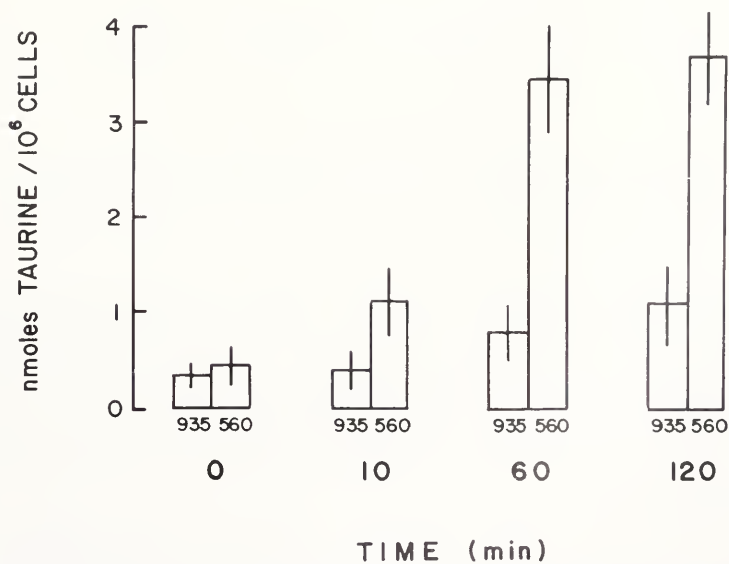


FIGURE 2. Taurine efflux from *Noctia ponderosa* erythrocytes in isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW. Vertical bars are ± 1 S.E.M.

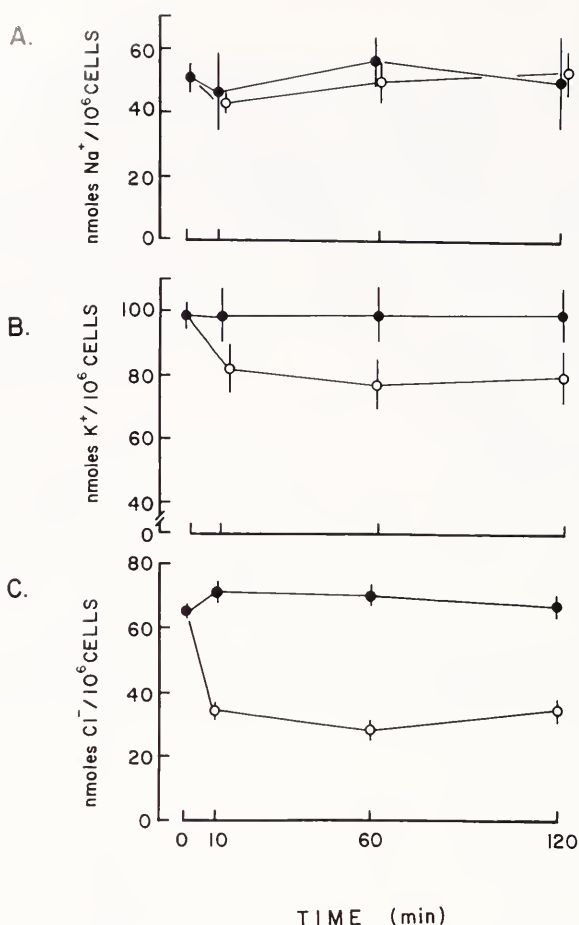


FIGURE 3. Intracellular Na⁺ (A), K⁺ (B), and Cl⁻ (C) content of *Noetia ponderosa* erythrocytes in isosmotic (closed circles) and hypoosmotic (open circles) ASW. Vertical bars are ± 1 S.E.M.

after hypoosmotic exposure, and little further decrease occurs subsequently (Fig. 3b). In addition, intracellular Cl⁻ decreases by 50% within 10 min of hypoosmotic exposure, from about 70 to 35 nmol/10⁶ cells (Fig. 3c). Thus, K⁺ and Cl⁻, but not Na⁺, leave *Noetia* erythrocytes after hypoosmotic exposure, and in contrast with the taurine efflux, this movement of intracellular ions begins immediately. The Cl⁻ efflux from *Noetia* erythrocytes was only partially balanced by the K⁺ loss. Thus, other cations besides K⁺ (but not Na⁺) must leave the clam erythrocyte during the early phase of volume recovery.

In the absence of Ca²⁺, the volume regulation in response to hypoosmotic ASW is partially inhibited (Fig. 1). Cells in the hypoosmotic Ca²⁺-free medium recovered slightly less than half the volume of control cells in normal hypoosmotic medium. Ca²⁺ lack did not affect the volume of erythrocytes in isosmotic ASW (Fig. 1).

Isosmotic Ca²⁺-free ASW had no significant effect on cellular K⁺ (Fig. 4a). Moreover, the usual hypoosmotic effect on cellular K⁺ content occurred whether or not Ca²⁺ was present in the medium (Fig. 4a). The effect of Ca²⁺ lack on intracellular Cl⁻ content was similar in pattern to that of K⁺. The Cl⁻ content of the cells was

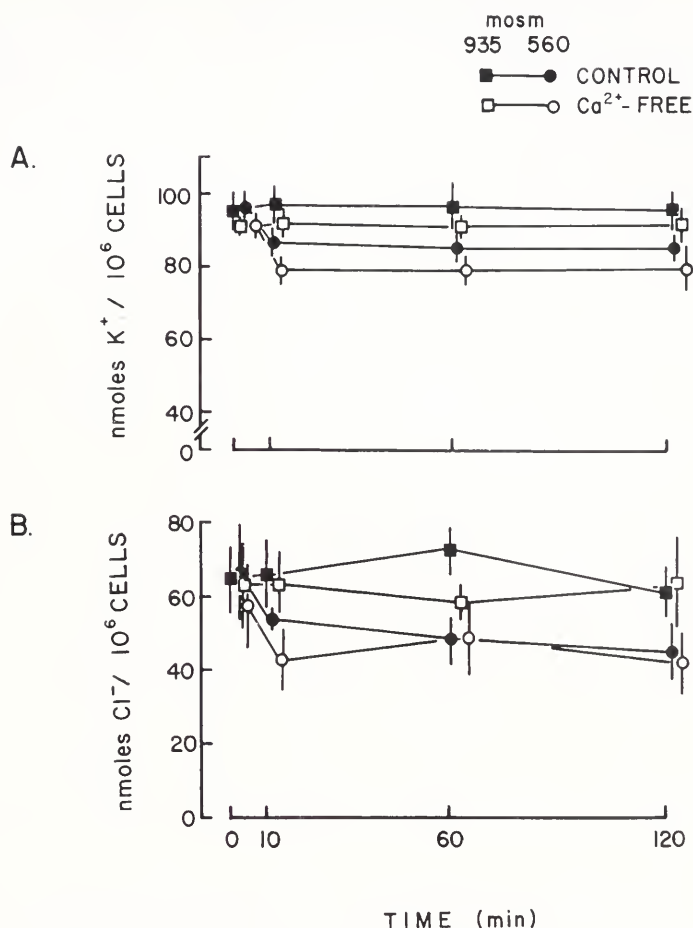


FIGURE 4. Intracellular K⁺ (A) and Cl⁻ (B) content of *Noetia ponderosa* erythrocytes in control and Ca²⁺-free isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW. Vertical bars are ± 1 S.E.M.

altered only by the usual amounts in response to the osmotic stress, regardless of [Ca²⁺]₀ (Fig. 4b).

In contrast, taurine efflux from *Noetia* erythrocytes in Ca²⁺-free hypoosmotic ASW was significantly less than that of cells in hypoosmotic ASW after 60 and 120 min (Fig. 5). A small yet significant efflux of taurine occurred from clam erythrocytes in Ca²⁺-free isosmotic ASW (Fig. 5). Therefore, taurine efflux was inhibited in Ca²⁺-free hypoosmotic ASW, while K⁺ and Cl⁻ effluxes were unchanged.

Cell volume regulation by clam erythrocytes in media containing elevated Ca²⁺ was potentiated (Fig. 1). Hypoosmotically stressed clam erythrocytes recovered to 119% of initial cell volume in the presence of high Ca²⁺, while the cells in hypoosmotic medium containing normal Ca²⁺ recovered to only 129% of initial cell volume. No significant change in erythrocyte volume occurred upon exposure of the cells to isosmotic high Ca²⁺ ASW.

High Ca²⁺ had no effect on taurine efflux from clam erythrocytes in isosmotic ASW (Fig. 7) or on erythrocyte K⁺ or Cl⁻ content in either hypoosmotic or isosmotic media (Fig. 6a, b). The usual decrease in intracellular K⁺ and Cl⁻ within 10 min

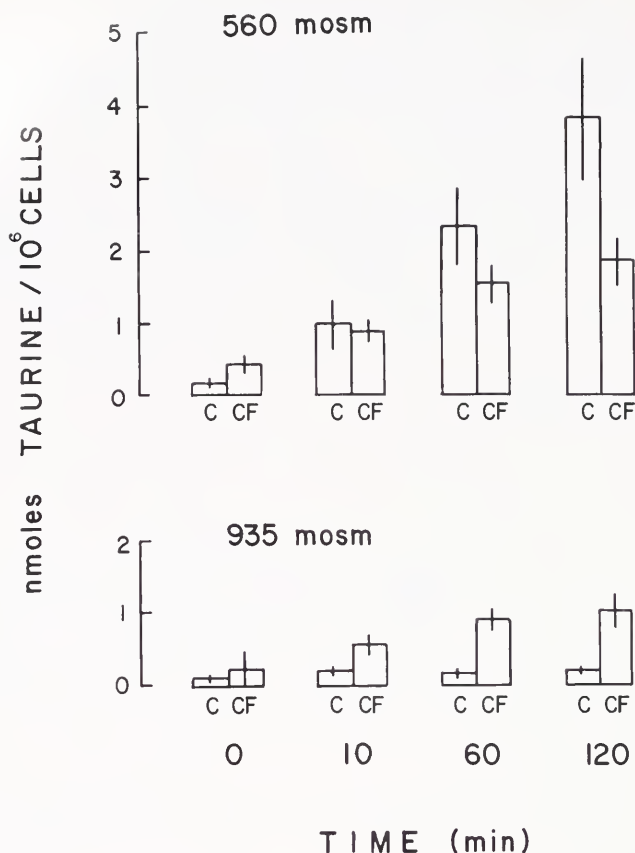


FIGURE 5. Taurine efflux from *Noetia ponderosa* erythrocytes in normal (C) and Ca^{2+} -free (CF) isosmotic (935 mosm) and hypoosmotic (560 mosm) ASW. Vertical bars are ± 1 S.E.M.

of exposure to hypoosmotic media (about 10 and 30 nmoles/ 10^6 cells, respectively) occurred in the presence of high Ca^{2+} (Fig. 6a, b). In contrast, the taurine efflux from *Noetia* erythrocytes in high Ca^{2+} hypoosmotic media was significantly greater, almost twice that of control cells (Fig. 7).

Thus, both Ca^{2+} -free hypoosmotic ASW and high- Ca^{2+} hypoosmotic ASW affect the volume regulation and taurine efflux, but not K^+ or Cl^- efflux, from clam erythrocytes.

DISCUSSION

Our results confirm our earlier report (Smith and Pierce, 1983) that cell volume regulation by *Noetia* erythrocytes in response to hypoosmotic conditions results from the efflux of both organic and inorganic osmolytes. *Noetia* erythrocytes use effluxes of K^+ , Cl^- , and amino acids to regulate cell volume during a hypoosmotic stress. The ion efflux occurs immediately in response to the stress while the efflux of taurine is delayed. Both cell volume regulation and taurine efflux are dependent on extracellular $[\text{Ca}^{2+}]$ but the ion efflux is not. Therefore, a mechanism is present which allows the two independent membrane permeability systems to produce a coordinated efflux of organic and inorganic osmolytes from *Noetia* erythrocytes. Both K^+ and Cl^- leave

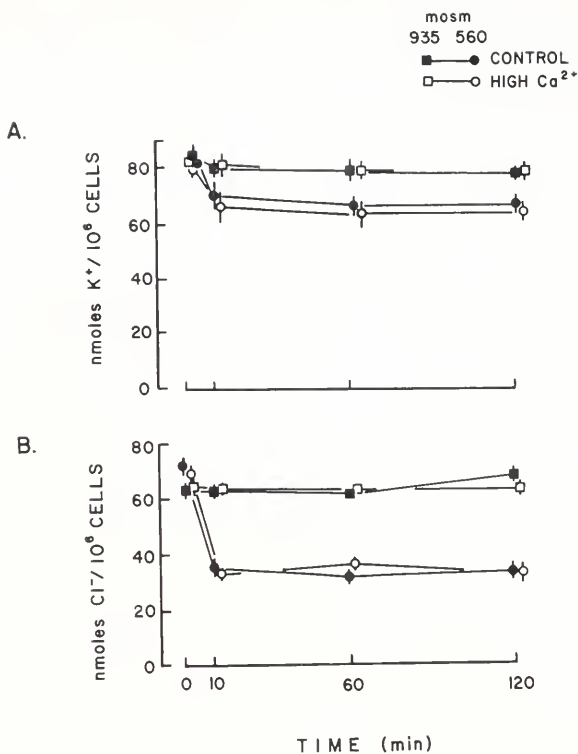


FIGURE 6. *Noetia ponderosa* erythrocyte intracellular K^+ (A) and Cl^- (B) content in control and high Ca^{2+} isosmotic (150 mM, 935 mosm) and hypoosmotic (90 mM, 560 mosm) ASW. Vertical bars are ± 1 S.E.M.

the *Noetia* erythrocytes immediately after the salinity stress while taurine concentrations do not change for many minutes. Thus, the two ions act alone as the osmolytes during the initial period of volume recovery. The amount of volume recovery resulting from the ionic efflux may be substantial. We do not know the maximum volume attained by the *Noetia* cells, but it is likely that volume recovery is well underway at our first sampling interval (5 min) (see, for example, Costa *et al.*, 1980). Therefore, the initial stage of ionic efflux accounts for most of the volume recovery.

The significance of a dual osmolyte efflux system is not yet clear, although it is not uncommon (Vislie, 1980; Costa and Pierce, 1983; Warren and Pierce, 1983; Moran and Pierce, 1984; Hoffman *et al.*, 1984). The differences between the ionic and organic osmolyte effluxes may provide a clue. Although the inorganic osmolyte efflux from the *Noetia* cells occurred immediately in response to hypoosmotic exposure, the concentrations of the ions may not remain reduced. In at least one case, after the initial reduction, the intracellular ion concentration was partially restored as the organic components were reduced (Warren, 1982). On the other hand, while the efflux of organic osmolytes is initiated later in the response, no restoration of pre-stress concentrations occurs. Although the data are limited, they suggest that the organic osmolytes are not functioning as effectors in direct response to the osmotic stress, but rather as osmotic replacements as the ions are regulated back to homeostatic levels. The organic osmolytes are kept at levels which produce cellular osmotic stabilization without the detrimental effects on cell functions that might be caused

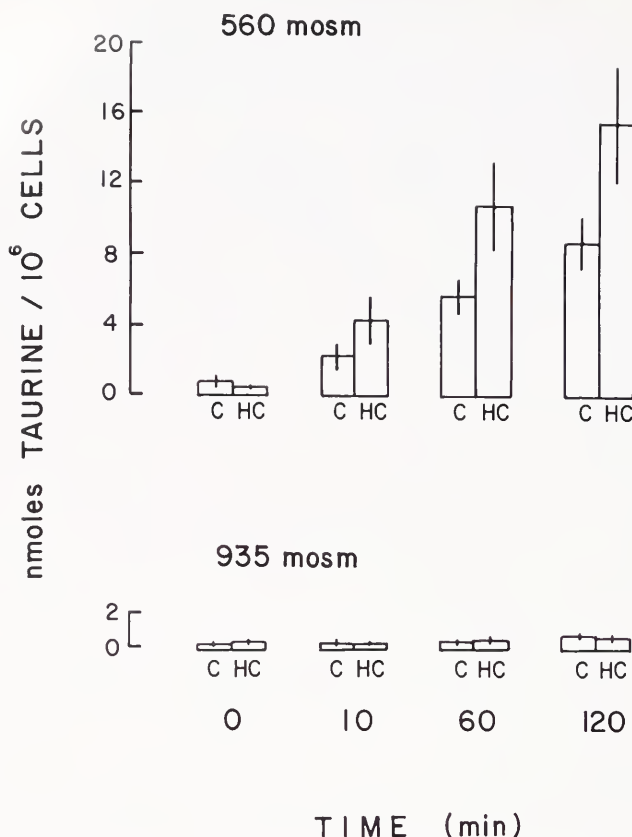


FIGURE 7. Taurine efflux from *Noetia ponderosa* erythrocytes in control (C) and increased Ca^{2+} (HC) isosmotic (150 mM, 935 mosm) and hypoosmotic (90 mM, 560 mosm) ASW. Vertical bars are ± 1 S.E.M.

by marked reduction in inorganic ion concentration. We are presently evaluating these ideas experimentally.

Our results indicate that the *Noetia* erythrocyte displays the components of volume regulation found among many other cell types. Rapid decreases of intracellular K^+ following hypoosmotic treatment occur in other invertebrate (Kevers *et al.*, 1979a, b, 1981; Costa and Pierce, 1983; Moran and Pierce, 1984) and vertebrate (Kregenow, 1971; Cala, 1977; Cheung *et al.*, 1982) cells. A hypoosmotically induced Cl^- efflux concomitant with K^+ release occurs in crustacean axons (Kevers *et al.*, 1979b) and teleost erythrocytes (Lauf, 1982). Cl^- loss is also associated with Na^+ efflux in *Limulus* myocardium (Warren and Pierce, 1982) and rat liver (van Rossum and Russo, 1984). Thus, the initial ionically based stage of hypoosmotic volume regulation in *Noetia* cells is qualitatively similar to the responses of many other cell types.

The hypoosmotically induced Na^+ and Cl^- effluxes from *Limulus* myocardium, like the ionic effluxes from *Noetia* blood cells, are unaffected by the absence of Ca^{2+} (and Mg^{2+}) (Warren, 1982). However, the ionic components of the volume regulating systems of some other cell types are sensitive to the ambient level of Ca^{2+} . For instance, the K^+ efflux from *Glycera* coelomocytes exposed to either isosmotic or hypoosmotic Ca^{2+} -free media is potentiated (Costa and Pierce, 1983). External Ca^{2+} is

important in the regulation of K^+ efflux from vertebrate cells as well. For example, K^+ efflux from amphibian red blood cells was inhibited in Ca^{2+} -free hypoosmotic media (Cala, 1983). Likewise, K^+ efflux and volume regulatory ability were impaired in Ehrlich ascites tumor cells (Hoffman *et al.*, 1984) and human lymphocytes (Grinstein, 1983) by Ca^{2+} -free conditions.

Both volume regulation and taurine efflux are inhibited in *Noetia* erythrocytes exposed to Ca^{2+} -free hypoosmotic ASW, and both are potentiated in high Ca^{2+} hypoosmotic ASW. Similarly, cell volume regulation is inhibited by Ca^{2+} -free hypoosmotic media in *Callinectes* axons (Gerard, 1975) and *Glycera* coelomocytes, although the FAA efflux from *Glycera* coelomocytes in divalent cation-free hypoosmotic media is potentiated (Costa and Pierce, 1983). In summary, the Ca^{2+} effects on volume recovery and the effluxes underlying it are not the same in every cell type. Ionic or organic osmolytes may be effected by Ca^{2+} change, but in the *Noetia* cells only the amino acid portion of the response is Ca^{2+} sensitive.

An additional aspect of Ca^{2+} regulation is suggested by the small amount of taurine released from *Noetia* erythrocytes in the Ca^{2+} -free isosmotic medium. A similar leak of amino acids occurred from *Glycera* coelomocytes in divalent cation-free isosmotic media (Costa and Pierce, 1983) and mussel ventricles (Pierce and Greenberg, 1973). The increase in FAA permeability in Ca^{2+} -free isosmotic media, in contrast to its decrease in hypoosmotic media, suggests that different Ca^{2+} sensitive mechanisms exist for the control of membrane permeability at normal and lowered osmotic concentrations. The increased membrane permeability to taurine caused by Ca^{2+} -free isosmotic media may be due to a lack of divalent cations that bind to and stabilize membranes (Lin and Macey, 1978; Papahadjopoulos, 1978; Swinehart *et al.*, 1980). But in any case, Ca^{2+} may have more than one action on osmolyte permeability depending upon the osmotic environment.

In conclusion, the characteristics of the volume response of the *Noetia* cells and their similarity to those of other cell types suggests that the clam red blood cell will be an excellent model with which to examine further effects and the roles of Ca^{2+} in the regulation of cell volume.

ACKNOWLEDGMENTS

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ABSTRACTS OF PAPERS PRESENTED AT THE GENERAL SCIENTIFIC
MEETINGS OF THE MARINE BIOLOGICAL LABORATORY
17-19 AUGUST 1987

Abstracts are arranged alphabetically by first author within the following categories: cell motility and cytoskeleton; comparative physiology; developmental biology and fertilization; ecology; integrative neurobiology and behavior; neurobiology; and vision. Author and subject references will be found in the regular volume index in the December 1987 issue.

CELL MOTILITY AND CYTOSKELETON

Ultrathin optical-sectioning-tomography achieved with the light microscope. SHINYA INOUÉ (Marine Biological Laboratory).

We have achieved optical sections as thin as 0.1 μm for rectified polarized light and phase contrast microscopy and 0.2 μm for DIC microscopy. Rapid, programmed, serial through-focusing is also possible. The conditions used to achieve the ultrathin optical sections were: (1) a new generation of well-corrected, high N.A. objective lenses (Nikon new series 100/1.4 and 60/1.4 and Zeiss Axiophot 63/1.4, Plan Apochromats); (2) a high N.A. condenser fully and uniformly illuminated with high intensity monochromatic (546 nm) light through a light scrambler (Ellis 1985, *J. Cell Biol.* **101**: 83a; Inoué 1986, *Video Microscopy*, Figs. III-21, III-22); (3) optical contrast enhancement and image correction with a condenser rectifier for polarization and DIC optics, together with improved lens coating; (4) analog enhancement with black level and gain controls on the video camera (DAGE-MTI 65M Newvicon); and (5) further digital enhancement (UIC Image-I). Optical sectioning, through-focal sectioning, recording, and analysis were also aided by (6) a solid, optical bench microscope with precision stage and drives (Inoué 1986, *ibid*); (7) stepper motor and timed controller for the fine focus; and (8) a 450 TV line-resolution laser disk recorder (Panasonic OMDR T2-2021 FBC). Examples of through ultrathin optical sectioning shown were: 5 nm immuno-gold stained mitotic microtubules, and prophase chromatin, in *Haemanthus*; Golgi-stained neuronal axon and dendrites; and surface filaments on buccal epithelial cells.

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Dynamics of spindle microtubules visualized in vivo by high resolution video polarization microscopy. SHINYA INOUÉ (Marine Biological Laboratory), EDWARD D. SALMON, AND LYNNE CASSIMERIS (Biology, UNC, Chapel Hill, NC).

We can now visualize individual microtubules (MTLs) directly in living cells using ultrathin optical sectioning in video polarized light microscopy. We report here the dynamic lateral association of MTLs seen in the mitotic spindle of newt lung epithelial cells using this new method. Throughout mitosis in the half spindle, two or more MTLs associate laterally over distances of 1 to 2 μm for 1 to 10 second durations, forming short-lived birefringent rods. In time-lapse recording, the 100 or so rods that stochastically appear in the 0.1 μm -thick optical section appear to shimmer (the northern lights flickering seen at lower resolution). In prometaphase, MTLs attached to the kinetochore form discrete, birefringent bundles, one to a kinetochore. The 0.2 μm -diameter bundle (of 10-20 MTLs) can be over 10 μm long or even reach the spindle pole. While the bundle is considerably more stable than the rods, it also changes length with time. Presumably as the extent of lateral association varies, the MTLs bundle or splay polewards. The MTLs may also assemble and grow, or disassemble and shorten. Each chromosomal spindle fiber (CSF) contains many more MTLs than those in the bundle; the dynamic lateral association of the kinetochore and non-kinetochore MTLs together contribute to the mechanical integrity and paradoxical lability of the CSFs. In anaphase the kinetochore MTLs are commonly not associated into bundles but fan out diffusely poleward. In late anaphase and telophase, individual waving MTLs and those splaying off the tips of the forming stem bodies are clearly seen between the daughter set of chromosomes. Birefringent actin filament (bundles) in the cleavage contractile ring and microvilli are also clearly visible.

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The heat-stability of squid axoplasm neurofilaments provides a rapid method for their purification. KENNETH S. KOSIK (Harvard Medical School) AND J. METUZALS.

A number of procedures have been used to purify neurofilaments from the squid giant axon. The polypeptides that are most consistently associated with the filamentous fraction are P60 (apparent Mr 60,000), P200 (apparent Mr 200,000), and a high molecular weight band (HMW) with an apparent Mr of ~400,000. Purification of these polypeptides includes cytochrome c precipitation, discontinuous sucrose-gradient centrifugation and gel filtration, and Millipore filtration. We find that the neurofilament polypeptides from *Loligo pealei* have heat-stability which permits their rapid purification.

The giant axon was dissected, the axoplasm extruded, and the cleaned sheath and axoplasm immediately frozen on dry ice. These tissues were homogenized in a microtubule assembly buffer (100 mM PIPES pH 6.6, 1.0 mM EGTA, 1.0 mM MgSO_4) with 2 mM phenylmethyl-sulfonyl fluoride and 5 mM EDTA. The homogenate was spun at $25,000 \times g$ for 15 min. The supernatant was made 0.4 M NaCl and immersed in a boiling water bath for 4 min. After boiling the tube was plunged into ice and spun at $100,000 \times g$ for 30 min. The supernatant was dialyzed and analyzed by SDS-PAGE.

Coomassie staining of the axoplasmic preparation revealed polypeptides of molecular weights consistent with the squid neurofilament proteins. The only contaminating bands were a group of polypeptides of Mr ~ 18,000 which may represent calmodulin. Trace amounts of identically migrating bands were present in the axonal remnant and sheath preparation. The putative P200 and HMW bands were stained by a squid neurofilament antibody directed against phosphorylated epitopes. Anti-IFA, which recognizes an epitope shared by all classes of intermediate filaments (Pruss *et al.* 1981, *Cell* 27: 419), immunoreacted with the putative P60 and P200, RT97 (Anderton *et al.* 1982, *Nature* 298: 84), which is directed against a phosphorylated epitope of mammalian high and mid-sized neurofilament, immunoreacts with squid P60, P200, and HMW. When mice were immunized with the heat-stable squid axoplasmic fraction, the resultant monoclonal antibodies were generally directed against both the P200 and HMW or principally against P60. The biophysical property of heat-stability suggests that the molecular structure of the neurofilament polypeptides is elongated with few hydrophobic domains relative to their mass.

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Dynamics of actin, myosin, and membranes in living cells during cell division. JEAN M. SANGER, JEFFREY S. DOME, BALRAJ MITTAL, AND JOSEPH W. SANGER (University of Pennsylvania).

The distribution of actin and myosin during cell division was followed in living cells by microinjection of fluorescent G-actin or fluorescent myosin light chains into PtK₂ cells. Cytoplasmic membranes were labeled by exposing the PtK₂ cells to the dye 3,3' dihexyloxacarbocyanine iodide. During prophase, the first stress fibers to disassemble were located over the condensing chromosomes. Stress fiber shortening accompanied the rounding up of the prophase cell. Fluorescent actin and myosin were concentrated in the mitotic spindle like all other fluorescent proteins that were injected into cells. During mid-anaphase, a dramatic accumulation of myosin and actin fluorescence occurred in a band beneath the membrane at the location of the former metaphase plate. The fluorescent band grew wider and brighter as cytokinesis began and persisted as a bright band until the terminal stage of cytokinesis when mid-body formation occurred. At this time two small bands of fluorescence formed at either side of the mid-body, perhaps acting as mini-cleavage rings that maintain the integrity of the mid-body during the spreading of the two daughter cells. Unlike actin and myosin, which were present in the mitotic spindle and cleavage furrow, membranes, as revealed by 3,3' dihexyloxacarbocyanine iodide, were absent from these structures. Observations of injected cells exposed to the dye showed no obvious concentration of membranes in the contractile ring. The striking exclusion of most membranous organelles from the mitotic spindle probably accounts for the concentration of contractile and non-contractile proteins that can be seen in the spindle when fluorescent analogues are microinjected into cells. We conclude that the actin-myosin contractile ring responsible for cytokinesis is formed not by the rearrangement of actin and myosin filaments already present in the cell cortex, but by a recruitment of these proteins from throughout the cytoplasm at mid-anaphase into a contractile band.

Calcium transients are required for mitosis. ROBERT B. SILVER (Laboratory of Molecular Biology, University of Wisconsin, Madison) AND SHINYA INOUE.

Mitosis is thought to be regulated by transient elevations of cytoplasmic Ca^{2+} concentration. This study tested directly if: (a) such Ca^{2+} transients occur during the cell cycle, and (b) if such Ca^{2+} transients are used by the cell to signal the initiation of Ca^{2+} -dependent biochemical pathways requisite for specific mitotic events [e.g., nuclear envelope breakdown (NEB), anaphase onset (AO)]. Such a model would require a source/sink for Ca^{2+} and a Ca^{2+} binding target within the cytoplasm. Therefore, inhibition of the

function of any one component in such a system should result in an arrest of Ca^{2+} -dependent steps in mitosis. An affinity purified antibody to the Ca^{2+} -pump of smooth muscle sarcoplasmic reticulum (SR) was prepared. This antibody inhibits ATP-dependent Ca^{2+} uptake by SR and mitotic apparatus (MA), and recognizes a single membrane protein of 105 kD in these membrane preparations. Microinjection of this IgG into second cell cycle *E. parva* blastomeres inhibits mitosis within two minutes of injection. Injections in mid-anaphase halt chromosome movement; there is a loss of MA birefringence. Injection of Ca^{2+} channel antagonists specific for endomembrane Ca^{2+} efflux channels 6–7 min prior to NEB or 2 min prior to AO prevents these events in a dosage-dependent fashion. Complete inhibition is achieved with 1 mg/ml TMB-8 or 7 $\mu\text{g}/\text{ml}$ ryanodine, consistent with their relative effectiveness upon isolated membranes. Thus, there are both a Ca^{2+} -pump and a Ca^{2+} channel required for NEB and AO. Existence of cytoplasmic sites which required Ca^{2+} -binding for activation of NEB and AO was tested by increasing intracellular Ca^{2+} buffering capacity by injecting increasing doses of antipyrilazo III (ApIII) ($\text{K}_D \text{Ca}^{2+}$ of $2 \times 10^{-8} \text{ M}$). Cells injected with 10 pl 0–30 μM ApIII continued normal mitotic cycling. Injection of 50 μM ApIII between 6 and 5 min prior to NEB, or 2 min prior to AO inhibited these mitotic steps. Injection of 40 μM ApIII was inhibitory in only 60% of the cases. The timing of the injections was critical—the sensitive period (and apparent Ca^{2+} pulse) occurring 6 min before NEB with a 40–45 s duration. This arrest by ApIII was not spontaneously reversible. This NEB arrest was reversible by a subsequent injection of CaCl_2 (10 pl 100 μM CaCl_2). The double injected cells did not resume cycling activity immediately, but underwent NEB and mitosis one cell cycle behind the control sister blastomeres. Injection of isoaequorins A, D, or E was used to detect directly these cytoplasmic Ca^{2+} . Cytoplasmic Ca^{2+} concentration drops as the cell enters mitosis. Transient elevations of cytoplasmic Ca^{2+} were found 6 min prior to NEB (40 s duration), 2 min prior to AO (10–20 s duration), and during cytokinesis. Rapid sampling methods demonstrated that these Ca^{2+} pulses were composed of rapid (8 s frequency) pulses. These results demonstrate that transient elevations of cytoplasmic Ca^{2+} are required for NEB and AO. They also suggest that the Ca^{2+} transients are used to synchronize parallel biochemical pathways required for NEB, AO, and mitosis.

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COMPARATIVE PHYSIOLOGY

A subarachnoid space in the elasmobranch brain—macro and microscopic evidence using large molecular weight fluorescent markers. ARTHUR M. BUTT (Dept. Physiology, School of Medicine, East Carolina University, Greenville, NC 27858).

The brain of elasmobranchs is classically considered to be enclosed within a single ill-defined *meninx primitiva* with only intraventricular (IV) cerebrospinal fluid (CSF). In fact, the skate (*Raja erinacea*) and the dogfish (*Mustelus canis*) appear to possess three meningeal layers analogous to the dura, arachnoid, and pia mater of higher vertebrates, and there is direct communication between the ventricular CSF and a subarachnoid space via a large pore in the posterior choroid plexus.

Fifteen minutes after injection of FITC-Dextran (MW 70 kD) intravascularly and Evans blue albumin (EBA, MW 60 kD) IV or subdurally, the brain was fixed and sections studied under the fluorescent microscope. EBA was observed to exit through the posterior pore and fill the subarachnoid space when injected IV, darkly staining the brain surface and subarachnoid blood vessels. There was further restriction to EBA movement between the subarachnoid CSF and brain tissue. There was no apparent loss of FITC from intracerebral or subarachnoid blood vessels. Also, EBA evidently diffused from the subarachnoid space down alongside larger vessels as they passed through the brain surface. EBA did not cross the arachnoid mater when injected IV or subdurally.

Elasmobranchs are therefore similar to other vertebrates, and have both arachnoid and dura mater with a subarachnoid space directly connected with the ventricular CSF and separated from the subdural space by a meningeal barrier. The perivascular space of intracerebral blood vessels is continuous with the subarachnoid CSF but is separated from the brain interstitial fluid by the blood-brain barrier. A barrier is indicated in subarachnoid vessels although its site was not discernible, and at the brain surface presumably at the level of the subpial glia.

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Effects of age on the skin pigmentation of the guppy (Poecilia reticulata). M. C. DICKMAN (Department of Biological Sciences, University of California, Santa Barbara, CA, 93106).

Development of color patterns and ultrastructural changes of the pigment cells that form these color patterns were studied in maturing male guppies using light and electron microscopy. Immature males were

tattooed for future identification and photographed using a stereo microscope for 2½ months. These were compared to photographs taken of fully mature males over the same time period.

Melanophores first appeared on the dorsal regions of the fish. As they matured, young fish exhibited a rapid migration of melanophores to all regions of their bodies. These melanophores formed a crisscross pattern one or two cells wide. They also formed massive aggregations in front of the caudal and behind the ventral fins. These aggregations developed into black spots. As the fish aged more spots formed on other regions of the body. Scales were removed from the spots and examined using a light microscope. These scales revealed large aggregations of melanophores lacking any distinct shape. In contrast, scales removed from non-spotted regions revealed melanophores of a stellate shape typical of teleost melanophores. The melanophores from scales of spotted areas did not respond when treated with epinephrine or atropine sulfate to induce aggregation or dispersion, respectively. When the melanophores from scales of other areas of the body were chemically stimulated, both aggregation and dispersion of the melanin granules were induced.

Electron microscopic studies of scale tissues taken from non-spotted regions of the body displayed morphological features typical of teleost pigment cells. In contrast, scale tissues taken from spotted regions revealed morphological features not found in scales of non-spotted regions. One type consisted of highly compressed aggregations of melanin granules embedded in an electron-dense cytoplasmic matrix. Another characteristic was an apparent disorganization of the cytoskeletal framework. Both electron microscopic and immunocytochemical studies are in progress to examine these characteristics.

Transection of the spinal cord near the obex abolishes cardiovascular compensation for gravity in bluefish (Pomatomus saltatrix). S. HUNTER FOX, CHRISTOPHER S. OGILVY, AND ARTHUR B. DUBOIS (John B. Pierce Foundation Laboratory, New Haven, CT).

The object was to determine if gravitational tolerance of the circulation of bluefish is abolished by transection of the spinal cord. Bluefish were lightly anesthetized with tricaine, placed on a V-board, and their gills perfused with seawater. Blood pressure (BP, mm Hg) was measured in the ventral aorta. The V-board was tilted head up in air at 10° or 20° for 2 min, and 30° for 5 min or 30 min. Before transection of the spinal cord, mean blood pressure decreased only slightly during the 10, 20, and 30° tilts. For example, in a group of 5 fish, a control BP of 52 mm Hg decreased to 43 SE 1.4 and heart rate (HR) increased from 53 to 68 per min SE 11.2 at one min of a 30° tilt. Transection of the cord at C-1, 7 mm caudal to the obex, did not alter the BP and HR response to tilting. Their BP decreased from 53 to 43 (SE 6.0) at 1 min of a 30° tilt and HR changed from 40 to 47 during the tilt. However, in 6 other fish, cord transection near the medulla (3 mm caudal to the obex) produced immediate tachycardia and a fall in BP during tilting to 10, 20, or 30°. After the cord section, BP fell from 48 to 38 SE 2.3 during a 10° tilt, from 43 to 33 SE 2.5 at 20°, and from 44 to 29 SE 3.0 at 30°. HR was 76 before and 78 during the 30° tilt. This region of the cord supplies sympathetic fibers to the heart and blood vessels in codfish [Nilsson, S. 1970 *Acta Zool. (Stockholm)* 57: 69-77]. We conclude that gravitational tolerance of the circulatory system in bluefish depends on integrity of the spinal cord and its adjacent nerves just caudal to the obex, but not on the integrity of the cord below the first cervical vertebra.

Initial results of lead measurements of deciduous teeth. ANN LEWANDOWSKI, MICHAEL RABINOWITZ, ALAN LEVITON, KIRSTEN IVERSEN, AND SUSAN ROSE (Neuroepidemiology Unit, Children's Hospital, Boston).

We have completed an analysis of 817 teeth as part of a prospective study of child development and environmental lead. These teeth are from children whose umbilical cord blood was measured for lead in 1979-80. These children represent a general population not especially at high risk for lead poisoning.

Lead tends to concentrate in calcifying tissues. In teeth, a portion of incisor dentine is mineralized concurrently with critical events of cortical development such as neuron migration and synaptogenesis; this portion may be sensitive to lead. From each tooth collected by mail, those portions of secondary dentine were sampled in duplicate (10 mg each), dissolved in acid, and analyzed for lead by anodic stripping voltammetry ($<0.05 \mu\text{g}$) under filtered air and with purified reagents. Recoveries of 0 to 100 nonograms of added lead were 100 ± 5 (SE) %.

We found an average of $3.8 \mu\text{g/g}$ (ppm) lead (SD = 2.5, range 0 to 45, 2% > 10 ppm). Blanks contribute about 0.2 ppm to these values; the average difference between the two splits of the same tooth are 1.05 ppm, and the mean difference between different teeth from the same child are 1.1 ppm (n = 24). Correlation of tooth lead and umbilical cord blood lead is 0.12 (Spearman r, $P = 0.02$). The tooth lead also correlates with the age of the tooth at shedding ($r = 0.17$, $P = 0.004$). As in cord lead, there are demographic trends in tooth lead relative to race and financial status. Thus, the tooth lead concentrations in this population, although low on average, are sufficiently variable to provide a retrospective marker of lead insult.

Inhibition by heparin of endotoxin-dependent coagulation of amoebocyte lysate from Limulus polyphemus. JAMES A. MARCUM (Harvard Medical School) AND JACK LEVIN.

Heparin is a highly sulfated complex carbohydrate composed of repeating glucosamine and uronic acid moieties. It exhibits potent anticoagulant activity in vertebrate clotting systems. *Limulus* amoebocyte lysate (LAL) contains a cascade of activators which generate a clotting enzyme that produces gelation of a clottable protein. We have examined the effect of heparin on endotoxin-dependent activation of LAL. Addition of commercially available (mammalian) heparin to LAL, prior to the addition of the bacterial lipopolysaccharide, inhibited coagulation, as determined by a gel end-point assay. Using a chromogenic substrate (S-2222) and diluted LAL (1/100), heparin inhibited activation of the lysate by endotoxin in a dose-dependent manner, with 50% of enzymatic activity inhibited by 2–3 μg uronic acid/ml. Addition of heparin after addition of the lipopolysaccharide to LAL did not inhibit the amidolytic activity of the clotting enzyme. The specificity of heparin inhibition was investigated by examining endotoxin-dependent activation of cell lysate in the presence of chondroitin 4-sulfate, chondroitin 6-sulfate, heparan sulfate, dermatan sulfate, and hyaluronic acid. Addition of each glycosaminoglycan (10 μg uronic acid/ml) separately did not inhibit LAL activation. To examine if the unique heparin structure that binds vertebrate antithrombin is responsible for the inhibition of LAL activation, the complex carbohydrate was fractionated into anticoagulantly active and inactive species, using antithrombin-sepharose. Both heparin species comparably inhibited lipopolysaccharide-dependent activation of cell lysate. The mechanism by which heparin inhibits LAL activation was examined by fractionating cell lysate into proactivator(s) and proclotting enzyme preparations with heparin-sepharose under endotoxin-free conditions. Studies conducted with the proactivator(s), proclotting enzyme, lipopolysaccharide, and chromogenic substrate revealed that heparin did not inhibit the activation of the proactivator(s) by endotoxin or its amidolytic activity, but that the complex carbohydrate did inhibit the generation of the clotting enzyme *via* the activator(s). The physiological relevance of the regulation of LAL coagulation by heparin has not been established.

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The antiquity of transglutaminase: an intracellular enzyme from marine sponge cells enhances clotting of lobster plasma. REED BROZEN, PETER SANDS, WILLIAM RIESEN, GERALD WEISSMANN, AND LASZLO LORAND (Marine Biological Laboratory).

The aggregation of dissociated cells of *Microciona prolifera* is the most ancient example (10^9 years) of stimulus/secretion coupling in multicellular animals. The cells secrete a species-specific aggregation factor in response to activators of protein kinase C and Ca ionophores (Weissmann *et al.* 1986 *Proc. Natl. Acad. Sci.* **83**: 2914–2918). The clumped cells in their matrix resemble a blood clot and we therefore determined whether sponge cells contained transglutaminase activity (Lorand and Conrad 1984, *Mol. Cell. Biochem.* **58**: 9–35). Whereas unbroken cells showed no such activity, high-speed ($150,000 \times g$, 60 min) supernatants of sonicated cells contained transglutaminase as judged by four criteria: (1) time-, concentration-, and Ca-dependent incorporation of ^{14}C -putrescine into N,N'-dimethylcasein; (2) incorporation of dansylcadaverine into dimethylcasein (0.3%) in agarose gels subject to electrophoresis; (3) clotting of citrated lobster plasma (gamma glutamyl-epsilon amino lysine crosslinking of fibrinogen); and (4) inhibition by primary amines (*e.g.*, histamine, dansylcadaverine) but not tertiary amines (*e.g.*, dimethyldansylcadaverine). The enzyme was concentrated over 25-fold from sponge cell sonicates and resolved on gel filtration chromatography (Sephadex G-100). Peak fractions were concordant for ^{14}C -putrescine incorporation and clotting of lobster plasma; both activities were inhibited by histamine and dansylcadaverine, but not dimethyldansylcadaverine. The sponge cells also contained an endogenous acceptor of ^{14}C -putrescine; incorporation was Ca-dependent and inhibited by primary amines. The data show that transglutaminase activity is present in species without blood, hemolymph, or closed coelom. The enzyme may play a role in the formation of new sponges from cell aggregates.

DEVELOPMENTAL BIOLOGY AND FERTILIZATION

The totipotent development of myoplasm-enriched ascidian embryos. W. R. BATES (Department of Zoology, University of Kyoto, Kyoto 606, Japan).

The rapid development of the ascidian larva (Urochordata) is thought to be controlled primarily by factors localized in the egg cytoplasm (Conklin 1905, *J. Acad. Nat. Sci. (Phila.)* **13**: 1–119). These factors

may be localized into distinct cytoplasmic regions of the fertilized egg by a series of precisely oriented movements termed ooplasmic segregation. The yellow pigmented cytoplasm—the myoplasm—is moved from its initial position in the egg periphery into the vegetal hemisphere. It is subsequently partitioned into the muscle and mesenchyme cell lineages by a determinant type of cleavage pattern. In the present investigation, fertilized eggs of *Styela plicata* or *S. clava* were bisected approximately along the equatorial plane into animal hemisphere fragments that lacked myoplasm and vegetal hemisphere fragments that contained all of the egg myoplasm. The egg fragments were cultured in pasteurized, millipore-filtered seawater until the controls reached the larval stage (18–20 h). Eighty-two percent (88/107) of the vegetal (myoplasmic) fragments cleaved while none of the anucleate animal (nonmyoplasmic) fragments cleaved. Twenty-two percent (24/107) of the vegetal fragments cleaved normally but arrested prior to gastrulation. Sixty percent (64/107) of the vegetal fragments developed into larvae (yellowhead larvae) in which myoplasm was present in many of the endoderm cells situated in the head region as well as in the tail muscle cells. In all of the yellowhead larvae tested, the pattern of expression of AchE activity (an enzyme marker for muscle cells; 22 tested), alkaline phosphatase (an enzyme marker for endoderm cells; 15 tested), and a muscle cell-specific antigen (detected by indirect immunofluorescence staining with a monoclonal antibody that was prepared by Mita-Miyazawa *et al.* 1987, *Development* 99: 155; 64 tested) was normal as compared to the controls. These results suggest that: (1) altering the distribution and concentration of the various kinds of egg cytoplasm into the embryonic progenitor cells before first cleavage does not affect the specification of larval cell types, despite Conklin's suggestion (1905) that the fertilized egg is comprised of distinct regions of tissue-specific morphogens and (2) a regulatory mechanism resides in the vegetal hemisphere of the egg that can compensate for the removal of the animal hemisphere without disrupting either the ascidian type of cleavage pattern or the specification of larval cell types.

Time dependent shift in fluorescence emission in gossypol treated Arbacia sperm. MARIO H. BURGOS (IHEM, U.N. Cuyo, Mendoza, Argentina) AND ROBERT B. SILVER.

Gossypol, the yellow pigment of the cotton seed, can produce sterility in mammals including human males, as described by the Chinese and confirmed by many investigators. The mechanism of action is not completely understood. However, gossypol is known to interfere with sperm motility and disturbs spermiogenesis and sperm maturation. The nature of the interaction between gossypol and macromolecules appears to be by a Schiff base formation and hydrophobic and nucleophilic attraction.

Two years ago we observed by epifluorescence that gossypol-treated *Arbacia* sperm acquire a rapid fluorescence which was difficult to analyze. With more adequate equipment we have analyzed this phenomenon.

Spermatozoa shed by nine-volt stimulation were suspended in filtered seawater and placed in wedge-sealed chambers. Spermatozoa attached to the cover glass were observed by an objective plano apo rectified $\times 100$ under immersion oil. After washing the chamber with filtered seawater (f.s.w.), pictures were taken with DIC and by epifluorescence microscopy every five seconds for one minute. Other chambers similarly prepared and washed with f.s.w. were filled with ($\pm$) gossypol, (–) gossypol, or (+) gossypol, each at a concentration of 100 μ Mol. The same procedure was applied to the three types of gossypol; the (+) and (–) are optical isomers of the racemic ($\pm$) gossypol.

During the first 5 seconds of exposure to the ($\pm$) gossypol and to the (–) gossypol, an orange-yellow fluorescence appears. In less than 10 seconds this color fades and is replaced by a stronger yellow fluorescence and then by a yellow-green one. The (+) gossypol did not produce fluorescence. The three gossypols stopped sperm motility and produced swelling of the midpiece.

A series of fluorescent pictures show a change in mitochondrial characteristics: a single fluorescent body changes into a group of fluorescent clumps.

This study concludes that (–) gossypol is the only fluorescent isomer. As such it acts like a fluorescent probe whose main target appears to be the mitochondrion. We postulate that the swelling of the midpiece may result from the fusion of gossypol with the plasma membrane phospholipids, causing alteration of its permeability.

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Phosphatidylinositol hydrolysis after Spisula oocyte fertilization. WILLIAM R. ECKBERG (Howard University, Washington, DC) AND ETE Z. SZUTS.

We recently reported evidence that activation of C-kinase is involved in GVBD in *Spisula* oocytes. C-kinase is normally activated in cells by a transient increase in the diacylglycerol content of membranes as a result of phosphatidylinositol hydrolysis. To test whether C-kinase could be activated in this manner in *Spisula* oocytes, we labelled oocyte lipids with [^{32} P]orthophosphate and determined the radioactivity in

various inositol lipids after fertilization or artificial activation by excess K^+ . Fertilization resulted in a rapid loss of radioactivity from phosphatidylinositol(4,5)bisphosphate (PtdInsP₂). Thereafter, the radioactivity in PtdInsP₂ increased and equalled the preinsemination value by 3 min after insemination. K^+ /activation resulted in a comparable, but more transient loss of radioactivity from PtdInsP₂. Furthermore, the radioactivity exceeded that in unactivated oocytes by 3–5 min after activation. Fertilization also resulted in a rapid, transient loss of radioactivity from phosphatidylinositol(4)phosphate (PtdInsP). However, K^+ activation did not affect the specific radioactivity of PtdInsP. These data indicate that (1) *Spisula* oocyte C-kinase can be activated by pathways similar to those which activate it in other eukaryotic cells and (2) that phosphatidylinositols are metabolized in both fertilized and K^+ activated oocytes, but their metabolism differs after fertilization or artificial activation.

Interaction of avidin with Spisula oocyte proteins. TATSUJI HANEJI (The Population Council) AND S. S. KOIDE.

Avidin is a protein present in avian egg white; it binds biotin. Biotin is a vitamin and a constituent of decarboxylases, enzymes involved in CO₂ production. This shows that *Spisula* oocytes contain avidin-interacting protein (AIP) and the reaction involves biotin.

Biotin-containing proteins were determined by a transblot method and identified by a cytochemical staining reaction using avidin-peroxidase. *Spisula* oocytes were homogenized and fractionated by centrifugation. The cytosolic proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose paper. The paper was treated with 4% BSA solution to block non-specific binding sites and incubated with avidin-peroxidase. The staining reaction was performed by development in 4-chloro-1-naphthol and hydrogen peroxide.

Intense staining occurred with two oocyte proteins with Mr of 70 and 118 kD. Proteins of other *Spisula* tissues showed none to minimal staining. The staining activity of avidin-peroxidase was blocked on incubating with biotin, demonstrating that avidin-protein interaction involves biotin.

To determine whether AIPs are related to oocyte maturation, *Spisula* oocytes were treated with 50 mM KCl to induce germinal vesicle breakdown. The staining reaction of the 70 and 118 kD proteins in the samples prepared from oocytes treated with KCl was intensified. The location of AIPs in oocyte cytoplasm was confirmed by a cytofluorescence technique using fluorescein isothiocyanate-avidin conjugate.

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Injected calcium buffers block fucoid egg development. L. F. JAFFE, M. H. WEISENSEE, AND J. E. SPEKSNIJDER (Marine Biological Laboratory).

Formation of a local growth zone in the developing fucoid egg may require the establishment of a zone of high cortical calcium in this region. To test and explore this hypothesis, we injected calcium buffers of the BAPTA type into six-hour-old *Pelvetia* eggs and observed their development. Such buffers should shuttle calcium from regions of high concentration to ones of lower concentration and thus flatten out and delocalize incipient gradients. Such a shuttle mechanism requires a buffer weak enough to release calcium at a sink as well as strong enough to pick it up at a source.

In fact, injection of critical intracellular buffer concentrations does reproducibly block the initiation of tip growth: such "baptized" cells may live for up to two weeks but they do not visibly differentiate or grow. The critical concentration varies with the calcium dissociation constant, or K_D of the buffer. So far, the higher the K_D and thus the weaker the buffer, the more effective it is: our weakest buffer—the newly synthesized 5,5' mononitro monomethylBAPTA with an intracellular K_D of about 30 μM —inhibits development down to a final intracellular concentration of only 30 μM ; our strongest—dimethylBAPTA with a K_D of about 0.4 μM —takes about 4 mM to inhibit; while dibromoBAPTA, with a K_D of about 4 μM , takes about 1 mM. This shows that the nitromethylBAPTA (K_D about 30 μM) is about 100 times as effective in inhibiting the initiation of tip growth than the stronger dimethylBAPTA (K_D about 0.4 μM).

These results support the calcium gradient hypothesis and suggest that the incipient tip region may contain free calcium at levels as high as 30 μM or more.

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Effect of UV irradiation on axis and muscle cell specification in embryos of the ascidian Styela. WILLIAM R. JEFFERY (University of Texas at Austin).

Eggs of *Styela clava* contain colored ooplasm which segregate into specific regions of the zygote following fertilization but before first cleavage. A yellow ooplasm, the myoplasm, first accumulates as a cap at the vegetal pole, then extends into a vegetal crescent, and eventually enters embryonic muscle cells.

The role of localized cytoplasmic region in axis and muscle cell determination has been examined by UV irradiation. In initial experiments, the entire surface of yellow crescent-stage zygotes was exposed to UV light. At a dose of approximately $330 \text{ ergs}/\mu\text{m}^2$, 100% of the embryos cleaved normally, but failed to complete gastrulation and arrested as radialized blastulae. Muscle cell development, as determined by a cloned muscle-specific actin probe, a muscle-specific monoclonal antibody, and acetylcholinesterase localization, was normal in the radialized embryos. The effective dose for elimination of muscle cell development was approximately $1000 \text{ ergs}/\mu\text{m}^2$. To determine the UV sensitivity periods for axis and muscle cell development, embryos were irradiated at various times between fertilization and gastrulation. Embryos irradiated between fertilization and the yellow crescent stage became radialized, whereas those irradiated after myoplasmic localization developed a normal axis. In contrast, muscle cell development was sensitive to irradiation throughout early development until about the 32-cell stage. In a second series of experiments, zygotes at the yellow cap stage were irradiated locally in either the animal or vegetal hemisphere. In general, UV light was more effective in suppressing axis and muscle cell specification when focussed in the vegetal hemisphere. The results suggest that the axis and muscle cells are specified by UV-sensitive factors which are localized in the vegetal hemisphere region of the zygote and function during distinct intervals of early embryonic development.

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Oocyte maturation-inducing substance (OMIS) in Spisula. A. L. KADAM (Population Council), S. J. SEGAL, AND S. S. KOIDE.

Full-grown *Spisula* oocytes are arrested at the dictyate stage of meiosis and undergo maturation following fertilization. Hirai *et al.* (*Biol. Bull.* **167**: 518) demonstrated that serotonin induces spawning and oocyte maturation in the surf clam, *Spisula solidissima*. Toraya *et al.* (in press, *Experientia*), reported that *Spisula* body fluid contains a substance that potentiates serotonin action in inducing oocyte maturation. The present study was undertaken to purify and characterize OMIS from *Spisula* body fluid and ganglion cells.

Spisula body fluid and nerve ganglion cells were collected. The ganglion cells were suspended in artificial seawater (ASW) and lysed by sonication. The body fluid and ganglion homogenate were filtered through a PM-10 membrane filter. The filtrate was adsorbed with activated charcoal and extracted with 0.1 HCl. The extract was concentrated and purified by HPLC using a reverse-phase ODS column, eluted with 0.1 N acetic acid containing 10% methanol. Maturation-inducing activity was assayed with washed oocytes and scored for germinal vesicle breakdown (GVBD) by light microscopy.

The HPLC purified substance obtained from the body fluid and ganglion cells induced GVBD in *Spisula* oocytes in a dose-dependent manner. OMIS is stable to trypsin and heat treatment, adsorbed by activated charcoal, and not retained on dialysis in a tubing with a mol. wt. cut off of less than 1000 daltons. Its retention time on HPLC elution is coincident with that of reference serotonin creatinine sulfate. In conclusion, OMIS is present in *Spisula* body fluid and in ganglion cells and is probably serotonin.

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Heterospecific sperm motility enhancement by sea urchin oocyte peptides. S. J. SEGAL (Rockefeller Foundation), M. H. BURGOS, S. INOUÉ, AND H. UENO.

Two sperm-activating peptides have been isolated from the egg jelly coat of *Hemicentrotus pulcherrimus* (Suzuki *et al.* 1981, *Biochem. Biophys. Res. Comm.* **99**: 1238-1244) and characterized as a decapeptide, Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly and a nonapeptide, Gly-Phe-Asp-Leu-Thr-Gly-Gly-Gly-Val. Samples of the polypeptides have been synthesized by R. Lundin, Pharmacia Co., Uppsala, Sweden, by Merrifield's solid-phase peptide synthesis procedure. We tested these substances (designated 7003 and 7203, respectively) for their ability to stimulate oxygen uptake and the speed of forward motion of sperm from the marine worm, *Chaetopterus pergamentaceus*. Oxygen uptake was measured with an oxygen electrode. For motility measurements, sperm were introduced into a wedge chamber and recorded with a $20 \times 10.50 \text{ NA}$ rectified objective lens in the presence of a $\lambda/10$ compensator, in a high extinction video polarizing microscope. The video signal captured on a Newvicon video camera was processed with the motion tracking function displayed every $1/5 \text{ s}$ by the Image-1 digital processor (Universal Imaging Co.). Washed *Chaetopterus* sperm moved at a speed of less than $100 \mu\text{m/s}$. The addition of either polypeptide at a concentration of $10 \mu\text{M}$ increases the speed of movement to $200 \mu\text{m/s}$ after 2 min of treatment. This elevated rate of movement was observed for 7 min. We conclude that sperm-activation polypeptides can accelerate sperm motility in a different species.

Evidence that a G-protein mediates 1-methyladenine induced maturation of starfish oocytes. FRASER SHILLING (Univ. of Southern California) AND LAURINDA A. JAFFE.

In response to 1-methyladenine (1-MA), starfish oocytes undergo germinal vesicle breakdown (GVBD) and initiation of meiosis. To investigate the possible role of a guanine nucleotide binding protein (G-protein) in transducing the hormonal signal across the plasma membrane, we microinjected oocytes of *Asterias forbesi* and *Asterias vulgaris* with the G-protein inhibitors guanosine-5'-O-(2-thiodiphosphate) (GDP- β -S) and pertussis toxin (PTX). GDP- β -S, a metabolically stable analog of GDP, binds to G-proteins and inactivates them. Similarly, PTX inactivates certain G-proteins by catalyzing their ADP-ribosylation. Thirty minutes after applying 1-MA (10^{-7} – 10^{-5} M), 100% of oocytes injected with control buffer had undergone GVBD ($n = 35$), compared to 4% of oocytes injected with GDP- β -S (2–4 mM final concentration in the oocyte, $n = 28$). Fifty minutes after 1-MA addition, only 36% of the GDP- β -S injected oocytes had undergone GVBD. PTX (2–6 μ g/ml final concentration in the oocyte) was also inhibitory. Prior to injection, the PTX was activated by heating with dithiothreitol at 35°C for 30 min; the control solution contained unheated PTX and dithiothreitol. Activated PTX or the control solution was injected into the oocytes, and 80 to 160 min later 1-MA (10^{-6} M) was applied. One hour after applying 1-MA, only 12% of the oocytes injected with activated PTX had undergone GVBD ($n = 25$), compared to 100% of the control injected oocytes ($n = 10$). The PTX was most effective if injected ≥ 80 min before 1-MA treatment, presumably because of the time needed to ADP-ribosylate the G-protein. Inhibition of oocyte maturation by GDP- β -S and PTX indicates the involvement of a G-protein in the coupling of 1-MA binding to initiation of meiosis.

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Entry of sperm into the animal pole of the egg of the ascidian Phallusia mammillata. J. E. SPEKSNIJDER, C. SARDET, AND L. F. JAFFE (Marine Biological Laboratory).

It has long been believed that sperm tend to enter near the vegetal pole of ascidian eggs (Conklin 1905, *J. Acad. Nat. Sci. (Phila.)* 13: 1–119). However, several observations have indicated that both animal and vegetal fragments of various ascidian eggs can be fertilized. We have reinvestigated this matter during a study on calcium waves at fertilization in ascidian eggs.

Unfertilized *Phallusia* eggs were preloaded with the Hoechst 33342 DNA dye (10 μ g/ml, 30 min), inseminated with preactivated sperm to ensure synchronous fertilization, and fixed in 5.6% formaldehyde in seawater as early as 6 s after insemination. Both the meiotic spindle at the animal pole as well as the nucleus of the sperm entering the dye-loaded egg become stained, and the angle between the two was determined. At 6 s after insemination, only 1–7% of the eggs are fertilized. Forty-two percent of these eggs show the sperm at an angle of 0–45° from the meiotic site, 38% at 45–90°, 17% at 90–135°, and only 3% at 135–180° ($n = 57$). This means that 80% of the eggs show the fertilizing sperm in the animal hemisphere. At 2 min after insemination, those percentages are 18, 25, 30, and 27, respectively ($n = 133$), and at 5 min they are 0, 6, 18, and 76 ($n = 59$), meaning that at this time the sperm is located in the vegetal hemisphere in 94% of the eggs.

We conclude that the sperm normally prefers entry into the animal hemisphere. The male pronucleus then moves towards the vegetal pole during the first phase of ooplasmic segregation, which occurs within the first few minutes after fertilization.

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Differentiation of Arbacia punctulata is blocked by the protease inhibitor leupeptin after fertilization. WALTER TROLL (NYU Medical Center) AND SARAH DAVIDSON.

Protease inhibitors such as leupeptin (Leup) acetyl-leucyl-leucyl-argininal, were shown to suppress transformation of NIH3T3 cells after transfection with the activated H-ras oncogene (Garte *et al.* 1987, *Cancer Res.* 47: 3159–3162). A possible mechanism of protease inhibitor action is interference of activation of DNA polymerase α necessary for specific DNA expression. Protease inhibitors are unique in only interfering in selective DNA expressions showing negligible general toxicity. We demonstrate that 50 μ g of Leup present 30 s after fertilization totally blocks differentiation of *Arbacia* to skeletal plutei. Leup partially interferes with plutei formation when added 7 or 12 h after fertilization but has no action, even at much higher concentrations, when added 20 h after fertilization. Plutei formation was usually observed at 24 h. Thus, the protease inhibitor Leup interferes with early genetic expression leading to differentiation and has no action after the genetic expression has occurred. Several proteases inhibited by Leup are candi-

dates for the genetic amplification mechanism. These include the trypsin type enzymes plasminogen activator, thrombin, and sulphydryl proteases such as calpain. Inhibitors of the trypsin class, tosyl-L-arginine methyl ester and 6-aminocaproic acid, interfered with plutei formation added 30 s after fertilization in millimolar quantities. In addition, the amino acid lysine in millimolar quantities interfered with plutei differentiation, while the amino acids arginine and ornithine had no effect. The mechanism of lysine interference with only negligible antiprotease action requires further study. It is tempting to speculate that lysine interferes with DNA expression by inhibiting ornithine decarboxylase-forming polyamines.

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Effects of gossypol analogs on Spisula sperm. H. UENO (Rockefeller University), C. PORTER, R. KAWASHIMA, M. H. BURGOS, K. WATANABE, S. J. SEGAL, AND S. S. KOIDE.

Gossypol, an anti-fertility agent for men, inhibits sperm motility. To evaluate its structure-function relationship, we prepared various derivatives and analogs of gossypol (16 compounds). Effects of these compounds upon sperm fertilizability, motility, and oxygen uptake were examined. The results obtained were compared with gossypol and the mechanism of action of gossypol was investigated.

Sperm fertilizability was examined by scoring the % GVBD of *Spisula* oocytes after incubation with sperm which was treated with the drugs. Four out of the 17 compounds (gossypol, gossypolone, 516, and 518) significantly inhibited sperm fertilizability. Sperm motility was examined by video-microscopy with special diffusion chamber designed by S. Inoué. Three of the above 4 compounds (gossypol, gossypolone, and 518) strongly inhibited sperm motility under the experimental conditions. Oxygen uptake of intact sperm in ASW (MBL) was measured by using a Clark type oxygen electrode in the closed chamber with constant temperature system. We found that gossypol increased the oxygen uptake with an optimum around 10–20 μ M. A similar observation was reported with isolated mitochondria. This phenomenon is known to be due to the uncoupling of oxidative phosphorylation. Only gossypol and a derivative with substitution at 6-OH groups act as an uncoupler. Neither gossypolone nor apo-gossypol show any uncoupling activity. The present findings suggest that there is no correlation between an increase in oxygen uptake and inhibition of sperm motility. Furthermore, the formation of hemiacetal, or the presence of aldehyde and 1-OH group is essential for the activity. Schiff base derivatives of gossypol are inactive. Uncoupling activity of gossypol is due to the oxidation of gossypol to gossypolone and this requires a free 1-OH group and a free 4-position to form quinone.

We now propose the following hypothesis as the mechanism of action of gossypol. Gossypol acts in two steps: (1) conversion of gossypol to gossypolone which consumes molecular oxygen leading to the reduction of oxygen tension in the sperm. (2) Subsequent metabolism of gossypolone to form dihydrogossypolone may be mediated with NADH. The net result is a lowering of the level of NADH in the mitochondria, and an interfering with the electron transport system. Both steps would inhibit sperm motility.

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Neural induction in ascidian embryos redivivus. J. R. WHITTAKER (Marine Biological Laboratory).

This study has reinvestigated neural induction (evocation) in the development of the ascidian larva as first described extensively by Reverberi and Minganti (1946, *Pubbl. Staz. Zool. Napoli* 20: 199–252; 1947, *Pubbl. Staz. Zool. Napoli* 21: 1–35). These authors observed that some brain tissues (and the melanocytes, found exclusively in the brain) originated in cell lineages derived from the animal-anterior (a4.2) cell pair of the bilaterally symmetrical 8-cell stage; expression of histological neural features depended on cellular interaction with endodermal-notochordal derivatives of the vegetal-anterior (A4.1) cell pair. Visible differentiation of melanized (black) melanocytes in the larva of *Ciona intestinalis* was used as the test system to reinvestigate these conclusions. Results of microsurgically isolating the blastomere pairs from the 8-cell stage and recombining them in various ways confirmed that one or more ectopically located melanocytes develop only in the a4.2/A4.1 combination of quarter embryos. No isolated quarter embryo by itself develops melanocytes, except very occasionally an a4.2 quarter embryo autonomously differentiates internally located melanocytes. Embryos were observed in culture for 24 h; *Ciona* larvae begin to hatch and are fully differentiated at 18 h (at 18°C). In addition, endodermal-notochordal enriched cells (the B5.1 pair) isolated from the vegetal-posterior lineage (B4.1) at the 16-cell stage and combined (at their "64-cell" stage) as a partial embryo with a4.2 cells (at the "16-cell" stage) act as an effective inducer of melanocytes in the combination. This finding illustrates that neural inductive properties are a general feature of endoderm-

notochord cell lineages and not solely a positional feature of the vegetal-anterior cells. The results of such a combination also confirm experimentally the conclusion of other investigators from various cell lineage marking experiments: A4.1-derived cells can not serve as precursors of brain melanocytes.

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ECOLOGY

Does copper affect the mating behavior of Gammarus annulatus Smith, 1873 (Amphipoda: Gammaridae)? GUSTAVO BISBAL (School of Oceanography, University of Rhode Island, Narragansett, RI 02882).

The effects of copper on the mating behavior of *G. annulatus* are examined. This species exhibits a precopula stage in which the female is grasped and held dorsally by the male and carried in this position for several weeks.

To investigate the way in which copper promotes pair separation thus diminishing the probability of reproductive contact, established pairs were exposed to three treatments during 96 hours: (1) control (filtered seawater); (2) 0.025 ppm of Cu and; (3) 0.1 ppm of Cu. In addition, the probable effect of Cu on preventing the precopula amplexus was tested on artificially separated pairs exposed to the same treatments.

Three 1.0 l plastic chambers, each with ten pairs, were used for each treatment. The medium was renewed every 12 h. At the same intervals the number of pairs in amplexus was recorded, dead animals were removed and sexed, and a water sample was taken for Cu (AAS) analysis, oxygen content, salinity, and pH measurements. Temperature was maintained at $20 \pm 1^\circ\text{C}$. Chambers were aerated constantly and a summer photoperiod was maintained. Animals were fed squid daily. In assessing behavior, mortality effects were removed by expressing the number of joined pairs as a percentage of the maximum possible number of pairs per treatment.

At 12 hours the number of observed pairs did not differ between initially joined and separated pairs, and the observed trend under each treatment showed no significant differences throughout the whole experiment. A significantly higher cumulative mortality was detected at 72 and 96 h for the 0.1 ppm treatment. However, mating behavior was unaffected by the copper concentrations evaluated under laboratory conditions, suggesting that the instinct for reproduction is manifested even under the chemically stressed conditions of this experiment.

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Relationship between trace metal distribution and sulfate reduction in surface sediment. PATRICIA M. A. BYRNE (Dept. of Oceanography, University College Galway, Ireland).

Fe and Mn hydrous oxide coatings on recently sedimented particles may be reduced beneath the sediment surface, releasing solubilized metals into pore waters. Conversely, dissolved Fe and Mn may be precipitated as hydrous oxides (oxidizing conditions) or very commonly as sulfides (reducing conditions).

To obtain estimates of trace metal distribution and rates of sulfate reduction with depth, two sediment cores were taken from Buzzards Bay (15 m), sectioned at 2-cm intervals, divided into several fractions, and anoxically incubated for 0, 1.5, 4, 7, 10, and 13 days. After incubation, pore water was extracted from the sediment for determination of sulfate, sulfide, Fe, and Mn concentrations.

Initial pore water sulfate concentrations were roughly constant to a depth of 18 cm; diagenetic modeling suggested that this feature must be due to infaunal irrigation of burrows. Sulfate reduction rates were highest (0.83 mM/day) at the surface and lower at all other depths ($\bar{x} = 0.097 \mu\text{M/day}$). Observed sulfide production was very low ($\bar{x} = 0.006 \mu\text{M/day}$) relative to the known sulfate reduction rate, implying sulfide precipitation; 98.9% of sulfur was either reoxidized to sulfate or incorporated into sulfide precipitates.

The most significant changes in metal concentrations occurred at the 0–2 cm interval. Pore water Fe and Mn increased after 1.5 days, probably due to reduction of Fe and Mn oxides. As sulfate reduction was initiated and sulfide produced, the metal concentrations decreased implying the formation of metallic sulfides.

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Effect of segment loss on reproductive output in Capitella sp. I (Polychaeta). SUSAN D. HILL (Michigan State University), JUDITH P. GRASSLE, AND MICHAEL J. FERKOWICZ.

Capitella sp. I and sp. II are considered the most opportunistic of the *Capitella* complex. They occur in the Woods Hole vicinity, but may have differential seasonal success in colonizing new areas. Regenerating worms are frequently found in collected samples. In experiments similar to those reported previously for *Capitella* sp. II, we investigated the effects of repeated tail removal (at 4-week intervals) and subsequent regeneration on reproductive output in *Capitella* sp. I.

At 20°C, *Capitella* sp. I females undergoing repeated amputation of posterior segments showed no significant reduction in the number of eggs produced per female until at least 4 weeks after the first amputation when compared with a matched sample of intact females. In comparison, *Capitella* sp. II females showed a significant decrease by the second week. In *Capitella* sp. I, the number of broods produced by regenerating and intact animals remained very similar for 6 weeks following first amputation. The observed reduction in total egg production resulted from a decrease in the number of eggs per brood.

In the reciprocal experiment, *Capitella* sp. I females at 20°C undergoing simultaneous reproduction and regeneration produced significantly fewer posterior segments in 4 weeks than a matched cohort in which reproduction was blocked by the absence of males.

Oosorption occurs in these species. We find that oosorption may occur in *Capitella* sp. I fed and starved regenerating females, and in isolated females in which normal spawning is blocked by the absence of a mate. The conditions leading to oosorption and the role of oosorption in providing resources for other processes are being investigated.

Support for this research was provided by NSF, OCE-8509169 (S.D.H.).

A green algal (chlorophycophytal) infection of the dorsal surface of the exoskeleton, and associated organ structures, in the horseshoe crab, Limulus polyphemus. LOUIS LEIBOVITZ¹ (Laboratory for Marine Animal Health, Marine Biological Laboratory) AND GREGORY A. LEWBART.

Known diseases of the horseshoe crab, including two blue-green (cyanobacterial) diseases, are reviewed. The results of a six-year (1981-1987) study of a previously unreported green algal infection of the exoskeleton in adult wild and captive horseshoe crabs are reported. Progressive chronic degenerative lesions in the dorsum of the exoskeleton, the eyes (ocelli, and large lateral eyes), the arthrodial membrane, and the base of the telson were observed. The disease often produced loss of tissue structures and functions, including shell deformities, abnormal molts, degeneration and loss of eye structures (including the cornea and ommatidia), erosion and perforations of the arthrodial membrane, and hemorrhaging from the heart. No loss of function could be attributed to telson lesions.

Direct microscopic studies of the green algae in the affected tissues and algal cultures revealed the young germlings' (zygotes) ability to extend their rhizoidal processes into and between the microscopic chitinous lamina that compose the horseshoe crab's exoskeletal surface structures and organs. This resulted in progressive tissue degeneration, necrosis, sloughing, and loss of tissue structure. Secondary bacterial and mycotic infections followed algal invasion into the deeper tissues.

Morphological and cultural studies of the green algal organism, at the light and electron microscopic levels, indicated that the pathogen belongs in the family Ulvaceae. Work is underway to further characterize the agent and the disease it produces, and develop methods for the prevention and control of the disease.

The results of this study were considered from the standpoint of the evolutionary development of a disease, comparative pathology, wildlife management, and management of laboratory colonies of horseshoe crabs employed for biomedical studies.

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The effect of the arborescent bryozoan Bugula spp. on the settlement, growth, and mortality of the colonial encrusting tunicate Botrylloides leachii. PHILIP E. MYERS (University of South Carolina).

Space is often seen as a primary limiting factor in fouling communities and I examined how *Bugula* maintains itself in these communities when it is a poor space competitor. Three treatments were used to

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examine if *Bugula* affected *Botrylloides*: (1) clean fouling plates (Alone); (2) plates with *Bugula* alone; and (3) plates in which the fouling community was allowed to grow and interact. For each treatment four 12 × 9 cm black plexiglas plates were hung 0.5 m below a floating dock in Eel Pond, Woods Hole. On alternate days the number of newly settled *Botrylloides* were counted and the area of the ten oldest colonies was measured to estimate growth. Excluding the first ten colonies that settled, all newly settled *Botrylloides* were removed. If a monitored colony was missing it was counted as a mortality. *Bugula* and Alone treatments had no mortality, while the whole fouling community had high mortality (77.5%). The high mortality was attributed to large settlement of the solitary tunicate *Mogula* spp. which covered most of the monitored *Botrylloides* colonies. *Bugula* seemed to have a strong positive settlement effect on *Mogula*, although it was quantified. Both settlement and growth were significantly different in all three treatments with Alone greater than *Bugula*, which was greater than the whole fouling community. The majority of the growth differences between Alone and *Bugula* treatments probably resulted from food competition, because *Bugula* cannot outcompete *Botrylloides* for space. Competition for food may be related to flow and differences in flow over a plate may be the cause of lower settlement in the *Bugula* treatment. Flow changes and the usurping of available space (95–100%) by *Mogula*, and overgrowth of existing colonies probably caused the fouling community treatment differences in settlement, growth, and mortality.

Image enhancement of wet seals on rocks and sand as the sample in population ecology of Phoca vitulina concolor and Halichoerus grypus, basic research for the closed model. DAVID PATON (Marine Biological Laboratory, Woods Hole, MA).

Population estimates of seals require a series of single observations. A vertical aerial photographic method has been standardized that allows investigators to classify the animals according to age and color without disturbing them. This method avoids distortion of the animals' resting spine length and provides resolution to 4 cm. The resolution is dictated by the altitude flown, film choice, and the focal length of the camera. This method, using a Cessna 172 aircraft and 70 mm format, has been developed for modest budget researchers, enabling them to participate in a western North Atlantic survey. Five days of coincident sampling must be synoptically charted. The catch sample is then repeated to confirm the number of seals.

Interviews conducted with prospective participants in the population survey require film processing, materials, cameras, training, and funds to cover costs of extra surveys. Color film (ASA 120) would be used with an intervalometer of the type designed at the University of Vermont, and flown with a boxed Pentax 250 mm camera fitted with a bubble level. The aircraft must be modified for noise reduction and camera port fitment. This fitting would require four shop hours. It would not require USA/FAA certification or defeat the status of the aircraft.

Forty-three gray seals were photographed on Wasque Shoal during a two-day lull between April storms last winter. Transparencies were back lit using an illuminated stage. Grain on the transparency was resolved using a 600× photographic microscope. Thirty-five millimeter slides were made at just above grain resolution using tungsten film matched to the irradiant temperature. Four yearling pups were detected. Enhancement of contrast using color filters revealed moult patches on the larger seals.

Effects of aerobic versus anoxic conditions on glutamine synthetase activity in Zostera marina roots: possibilities for regulation of ammonium assimilation. A. MARSHALL PREGNALL (Vassar College).

Eelgrass (*Zostera marina* L.) assimilates ammonium from sediment pore water via the enzyme glutamine synthetase (GS) in the roots. During summer, plants growing at depth have greater GS activity than plants growing in shallow water. Sediment-free plants were held in continuous light for several days, causing prolonged root aerobiosis; root GS activity decreased below that of control plants held under normal light-dark cycles. In contrast, plants held in sediments for several days in continuous darkness, causing prolonged root anoxia, showed much increased root GS activity. This increase may result from *de novo* synthesis of GS or from feedback effects of different amino acids and adenylates that fluctuate with root anoxic/aerobic conditions. One hour pretreatment of root extracts with alanine (1–25 mM), which accumulates in anoxic roots, only slightly inhibited GS activity; γ -amino butyric acid, which also accumulates during anoxia, enhanced GS activity. Pretreatment with glutamate or glutamine (1–25 mM), which accumulate during root aerobiosis, also increased GS activity. Pretreatment with ATP (1–10 mM) slightly decreased subsequent GS activity, while pretreatment with AMP and ADP enhanced subsequent activity. Excised roots were incubated with cycloheximide (1–20 ppm) while under nitrogen for two days; GS activity was lower than in roots without cycloheximide, suggesting that part of the enhancement of GS activity during extended root anoxia results either from *de novo* synthesis of GS molecules or from some other protein-synthesis-dependent process. Thus it appears that hypoxic/anoxic root conditions produce changes

in both metabolite levels and protein synthesis that enhance the ammonium assimilation potential of *Zostera marina* roots following the return to aerobic conditions.

The chloroplast-retaining ciliates as a component of the primary productivity in Great Harbor, Woods Hole, Massachusetts. MICHAEL S. ROBERTS (Department of Biology, Wesleyan University, Middletown, CT 06457).

Certain marine planktonic ciliates have been known since the late nineteenth century to sequester chloroplasts from microalgae, but functional photosynthesis by chloroplast-retaining ciliates has only recently been demonstrated. As planktonic ciliates, primarily tintinnids (Order Choreotrichida) and oligotrichs (Order Oligotrichida), are an important component of the microplankton biomass in coastal waters, it is possible that mixotrophic ciliates which contain functional chloroplasts comprise a significant component of the neritic primary productivity. To determine the significance of ciliate-retained chloroplast productivity, water samples were collected from Great Harbor at the surface, 1.5, and 3.0 meters depth in early August. In surface waters, 43% of the planktonic ciliates possessed chloroplasts although the surface water chlorophyll *a* content was only $1.2 \text{ mg} \cdot \text{m}^{-3}$. At 1.5 and 3.0 meter depths, chloroplast-retaining ciliates comprised 37% and 58% of planktonic ciliate density, respectively. These deeper neritic waters possessed an average chlorophyll *a* content of $1.3 \text{ mg} \cdot \text{m}^{-3}$. Measurements of primary productivity by means of ^{14}C uptake and Winkler titration indicated a net photosynthetic deficit in surface water and water at 3.0 meters depth. Net photosynthesis exceeded respiration in water at 1.5 meters depth, reaching a value of $30 \text{ mg} \cdot \text{C} \cdot \text{m}^{-3} \cdot \text{h}^{-1}$. Although the chloroplast-retaining ciliates remain an important component of the planktonic biomass in late summer, their contribution to community primary productivity is apparently negated by the increased density of heterotrophic ciliates.

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Nitrate reductase activity in Zostera marina. NINA C. ROTH AND A. MARSHALL PREGNALL (Vassar College).

Nitrate reductase (NR) activity in *Zostera marina* was investigated using an *in vivo* assay; the optimal incubation medium contained 60 mM nitrate, 100 mM phosphate, and 0.5% 1-propanol at pH 7.0. *Zostera* leaves showed significantly higher NR activity than roots [350 nmoles nitrite/(g FW*h) versus 50 nmoles nitrite/(g FW*h)]. The effects of depth (deep, middle, shallow) and location within the eelgrass meadow (patch edge versus middle) on NR activity were examined using plants collected from three locations in Woods Hole, Massachusetts. Nitrate enrichment experiments (200 μM NO_3 for six days) were conducted to determine if NR activity can be induced. Results indicate that neither depth nor location within the patch significantly affect nitrate reductase activity. Certain plants (shallow, 1.2 m depth) did respond to the enrichment treatment, showing a significant increase in nitrate reductase activity over six days [<100 –950 nmoles nitrite/(g FW*h)]. It appears that shallow edge *Zostera* plants growing near a shoreline may be affected by run-off or ground water percolation, since plants from this area exhibited rates up to 1600 nmol nitrite/(g FW*h). Water samples from this location revealed higher nitrate concentrations (1.35 μM NO_3) than other collection sites (0.7 μM). Thus, it is possible that chronic run-off causes sufficient nitrate enrichment in the water column to induce nitrate reductase activity in *Zostera* leaves.

An estimate of primary productivity in Waquoit Bay National Estuarine Sanctuary, Falmouth, Massachusetts. EDWARD T. ROWAN (Science Department, Falmouth High School).

Waquoit Bay National Estuarine Sanctuary for Research and Education is located in Falmouth, Cape Cod, Massachusetts. The watershed of this restricted opening bay is prime real estate for development. A survey of this area was initiated so that students at Falmouth High School might participate in monitoring the bay.

Eight stations within the bay were compared to a station established in Vineyard Sound. Consideration of freshwater inputs and exchange with Vineyard Sound led to the positioning of the stations. A bouy capable of floating at least four 300 ml glass BOD bottles was anchored at each station by two cement construction blocks. All stations were sampled for temperature, salinity, dissolved oxygen, and pH at 0.5

meter intervals through the water column during differing tidal phases to determine the degree of mixing. A transect for salinity was taken along the channel to assess the exchange of water with the sound.

Primary productivity experiments were performed at each station. Two sets of light-dark bottles were suspended in the mid-water column. All bottles were filled with mid-column water; one set was enriched with nutrients. All samples were incubated for four hours. A sample was returned to the lab for the determination of concentration of chlorophyll *a*. Light attenuation coefficients were calculated for each site.

Net productivity was generally low, as was the concentration of chlorophyll *a*. The nutrient-enriched samples were more productive than those without this treatment. Two stations showed increased net productivity that may be linked to higher levels of chlorophyll *a*. These stations do not appear to be nutrient-limited and are located closest to the major freshwater inputs. These data lack the fidelity that would come with increased samples.

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Don't eat if Neptune is angry. BERND SCHIERWATER AND GEOFF TRAGER (Marine Biological Laboratory, Woods Hole, MA 02543).

The medusa stage of the hydrozoan *Eleuthera dichotoma* is very unusual in its biology compared to other hydromedusae. This tiny medusa (umbrella diameter less than 0.5 mm) lives half-sessile in shallow coastal waters and is not capable of swimming. In respect to the generally high water velocities in this environment, a half-sessile lifestyle poses the risk of being detached by currents. In this study, we investigated this organism's ability to remain attached to the substrate in high speed currents.

A special laser optical path and videorecording were used to determine water velocities at the moment of detachment. We found statistically significant differences in ability to resist detachment between fed and nonfed medusae. At water velocities ranging from 24 to 70 cm/s we could detach 87% of the freshly fed medusae. Whereas only 25% of nonfed medusae could be detached at water velocities from 33 to more than 70 cm/s. The results indicate that because of drastically increased surface area after feeding, drag forces necessary to remove medusae are achieved at lower velocities. The range of calculated drag forces at detachment for fed medusae was 3.6×10^{-6} to 31×10^{-6} Newton. Videotaping also showed that under conditions of high water velocities the medusae move their body center down to the bottom to come into the slow-moving boundary layer.

E. dichotoma medusae seem highly adapted to a half-sessile lifestyle, being capable of holding on to the substrate at very high velocities. Feeding also significantly increases the risk of being detached.

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Protein synthesis and degradation rates in two ecophenotypes of the cord grass Spartina alterniflora Loisel from Great Sippewissett Salt Marsh, New England. KRISHNAN THANKAVEL (Centre of Advanced Study in Marine Biology, Annamalai University, Portonovo 608 502, Tamil Nadu, India), MARSHALL PREGNALL, AND KANNUPANDI THADAMA.

The salt marsh cord grass, *Spartina alterniflora* Loisel, is the most productive of the marsh grasses and has two ecophenotypes, viz. tall form and short form. This study attempts to estimate the rates of protein synthesis and protein degradation in *S. alterniflora* and their role in the nutrient cycle in a salt marsh. Young leaves at the moment of their expansion and old leaves were detached and placed in the dark. Protein and chlorophyll *a* contents and the rates of protein synthesis and degradation were measured at different times from 0 to 4 days after detachment. Rates of protein synthesis were measured by the incorporation of [4,5-³H] leucine into proteins. Rates of protein degradation were determined from the disappearance of radioactivity from proteins already labeled with [4,5-³H] leucine.

The short form of *S. alterniflora* had more protein than the tall form and concentrations of protein increased as the leaves senesced. Chlorophyll *a* decreased with time. Old leaves of the tall form of *S. alterniflora* took up the maximum tritiated leucine on the third day after detachment. In the short form the young leaf took maximum leucine one day after detachment. Measurement of degradation indicates that unlike most other plants, young and old leaves of both ecophenotypes of *S. alterniflora* synthesize and accumulate protein until three days after detachment. Thus, *S. alterniflora* leaves may act temporarily as a nitrogen sink or trap which contributes to the nitrogen limitation in the salt marsh.

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Crocker Fellowships to T. Krishnan at the Marine Biological Laboratory, Woods Hole, USA. A very special thanks to the staff and students of the Marine Ecology Course.

Life is rough when you are small. GEOFF TRAGER (Marine Biological Laboratory, Woods Hole, MA).

Small sessile suspension feeders in aquatic environments are faced with certain physical problems with respect to size. In low Reynold's number environments, water is viscous, and small organisms such as bryozoans and newly settled barnacles must spend considerable energy to move water past their feeding structures. In this study a special laser optical pathway and video recording were used to analyze the relation between structure, behavior, and water velocity in both large and small barnacles.

Results indicate that barnacles feed actively in slow currents by raking the water with cirri but change to passive suspension feeding, where cirri are held stationary in the current, at higher velocities. This switch occurs in a specific flow speed range. The range is lower for big barnacles than for small ones. Because of small-scale viscous and boundary layer effects, big barnacles appear to be able to take advantage of energy in ambient currents more readily than small barnacles. Results show barnacles to be sensitive to changes in water velocity, and to be well-adapted for suspension feeding in a fluctuating flow environment.

I thank Dr. J. R. Strickler for providing laboratory space, special optical equipment, and useful discussion.

Bacterial uptake of glutamic acid in oxic and anoxic waters in a coastal pond. DIANA E. VARELA (Centro Nacional Patagonico, Puerto Madryn, Argentina) AND JOHN HELFRICH.

Heterotrophic activity of bacteria in aquatic environments uses dissolved organic compounds to produce particulate organic material which forms the basis of the food chain. The bacterial activity at different depths can be compared by measuring the turnover time and the rate of uptake of a dissolved amino acid such as glutamic acid.

Measurements were made on samples from Siders Pond, a brackish water coastal pond with no river input and a stable stratification. As a result, the bottom layer is anoxic and contains high concentrations of nutrients. Glutamic acid labeled with ^{14}C was added to water samples to observe the amino acid incorporation and respiration rate. The results were analyzed by Michaelis-Menten kinetics equations.

The depth profile of glutamic acid uptake was determined in samples collected at 2-m intervals. Deeper samples were maintained under an N_2 atmosphere to preserve anoxic conditions. The profile showed greater uptake in the layer above 10 m. Accordingly, samples for time series and kinetics measurements were taken at 2 and 12 m.

The time series experiment showed similar turnover times for the bacterial populations from both depths (3.2 h for 2 m and 2.9 h for 12 m). The kinetics experiments confirmed these results and yielded a ten-fold greater maximum uptake velocity at 2 m (67 versus 6 nmoles/l·h). The sum of the uptake constant and the concentration of naturally occurring substrate ($K + S$) was higher at 2 m than at 12 m (155 nmoles/l versus 20 nmoles/l).

These results demonstrate that the bacterial population at 2 m turns over a greater quantity of dissolved amino acids than that at 12 m.

D.E.V. thanks John Hobbie and Ivan Valiela for their patient guidance and the staff and students of the Marine Ecology Course at the Marine Biological Laboratory for their constant help. Funds were awarded for this project from the Frank R. Lillie Fellowship.

Co-selection for clumping and phosphorus accumulation by bacteria isolated from waste-water treatment systems. W. S. VINCENT AND SARA PRATT (University of Delaware).

Previous studies described the clumping behavior of strains of bacteria isolated from a waste-water treatment system, and showed that the system acts as an artificial ecosystem which imposed rigid selective restraints on the component bacterial populations. The ability to form clumps is one of the traits which is selected. Other characteristics of the bacterial system which are obviously required are oligotrophy and facultative anaerobiosis.

Sludges formed in the waste-water system studied (Air Products and Chemicals, Inc. patented A-O system) also contained 5–10% dry weight concentrations of polyphosphate. The selective value of this trait is not immediately obvious. This led us to examine the phosphorus accumulating properties of 34 different strains (non-*Acinetobacters*) isolated from A-O sludges. All of the 20 different clumping strains in this set

accumulate phosphorus to some degree with 10-fold variations among the strains in both dilute and normal culture media where phosphorus was not limiting. In media designed to stimulate maximum phosphorus accumulation, 6 of the strains contained more than 10% dry weight of P. Overall, the mean P content of the 20 strains in this medium was 7.1%, approximately the average of the *in situ* system sludges.

We conclude that clumping and the ability to take up and store P are co-selected in many species in this system.

INTEGRATIVE NEUROBIOLOGY AND BEHAVIOR

Signalled avoidance learning of eye withdrawal in the green crab is predominantly Pavlovian in mechanism. CHARLES I. ABRAMSON, PHILIP M. ARMSTRONG, ROBIN A. FEINMAN, AND RICHARD D. FEINMAN (SUNY Health Science Center at Brooklyn, Brooklyn, NY 11203).

Signalled avoidance learning was readily demonstrated in the green crab, *Carcinus maenas*. A mild vibration to the carapace served as a warning (conditioned) stimulus (CS). Eye withdrawal during the CS led to the omission of an otherwise scheduled puff of air to the eye (US). Acquisition was rapid, reaching about 75% avoidance after about 30 trials. Extinction occurred slowly over the course of 40 CS only trials. The comparative analysis of learning has shown that at least two underlying mechanisms, one Pavlovian and the other operant, can govern the performance of animals in signalled avoidance situations. To determine the mechanism involved in learning in the crab, additional groups of animals were subjected to (1) a classical conditioning paradigm in which CS responses had no effect on US presentation, (2) avoidance (60 trials) followed by classical conditioning (40 trials), or (3) classical conditioning followed by avoidance. The behavior of all groups was essentially identical indicating that the association between the CS and US was sufficient to effect conditioning and that the animals were unaffected by the consequence of their actions. In this they resemble some avoidance behaviors such as the nictitating membrane response of the rabbit or shuttle box performance in teleost fish, which show predominantly Pavlovian behavior, rather than the shuttle box avoidance in rats where true operant avoidance has been demonstrated.

Functional organization of the sonic motor system in sea robins. ANDREW BASS (Cornell University, Ithaca, NY), MICHAEL WEISER, AND ROBERT BAKER.

Sea robins generate sounds by contracting a set of bilateral 'sonic' muscles that are intrinsic to the walls of the swimbladder. Each muscle is innervated ipsilaterally by a sonic motor nucleus (SMN) that lies along the ventrolateral margin of the caudal medulla and rostral spinal cord. Bipolar stimulating electrodes were implanted in each of the sonic muscles to antidromically activate its respective SMN. Individual motoneurons were identified on the basis of the timing of action potential generation relative to outputs from the sonic nerves as monitored with bipolar silver ball electrodes placed intracranially upon ventral motor roots that arise from the SMN. Intracellular injections of horseradish peroxidase (HRP) demonstrated that motoneurons typically have a teardrop-shaped soma with a small basal dendritic tree and a large apical dendrite which ascends dorsolaterally to end in a large dendritic tuft. A single, unbranched axon exits via a ventral root. There was no evidence for any direct connections between the two sonic motor nuclei.

Central stimulation in the midbrain, using brief trains of low amplitude pulses, evoked an asynchronous response in the ventral roots similar in frequency and duration to natural vocalizations of 100–200 Hz. We conclude that the distinctly bilateral sonic motor system in sea robins is functionally correlated with the asynchronous firing of the two sides of the swimbladder. By firing the two sonic muscles at the same frequency but out of phase with one another, sea robins can effectively double the fundamental frequency of their 'vocal' signals. The location and properties of the pacemaker neurons that determine the degree of asynchrony in firing between each SMN remain to be shown.

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Extrinsic optical signals, evoked field potentials, and single unit recordings from the olfactory bulb of the skate (Raja erinacea). A. R. CINELLI AND B. M. SALZBERG (University of Pennsylvania).

We have used multiple site optical recording of transmembrane voltage (MSORTV), together with conventional electrophysiological techniques to study electrical activity in various layers in the *in vitro* and

in vivo olfactory bulb of the Atlantic skate *Raja erinacea*. In the living animal, orthodromic stimulation evoked field potentials characterized by an initial triphasic wave, reflecting the compound action potential in the olfactory nerve, followed by negative waves (N1, N3, N4, and, somewhat variably, N2). The current generators for these waves were studied by laminar and current-source density analyses. N1 corresponds to the excitation of the glomerulus, N3, depolarization of granule cells, and N4, probably reflects the re-excitation of mitral cells.

Paired stimuli were used to study the properties of these waves. The N1 wave first exhibits a period of facilitation, followed by a period of partial suppression. N3 has a period of nonlinear suppression. N4 exhibits a long-lasting suppression with a duration greater than 2 s.

Single unit discharges, presumably from mitral cells, also exhibited a period of suppression of the test response, following paired stimuli. Some neurons showed a labile period of re-excitation. Late evoked responses with similar properties were also seen.

Optical recordings of electrical activity from 500 μm slices of skate olfactory bulb were obtained using the extrinsic absorption changes exhibited by the pyrazo-oxonal dye RH 155. Following orthodromic stimuli, two depolarizing responses were evoked. A fast signal, observed in the upper region of the glomerular layer, represents the compound action potential in the olfactory nerve. A signal of longer duration was observed in the zone between the glomerulus and the mitral somata, and in deeper layers. Both signals exhibited a wavelength dependence characteristic of RH 155, and were abolished in the presence of 2 μM TTX. Cadmium (100 μM) eliminated the slow component without affecting the fast signal, suggesting that the former is synaptically mediated. Barium (5–10 mM), which depolarizes glial cells, increased the size of the slow component, suggesting that this optical signal does not reflect a glial response to $[\text{K}^+]_o$. Different condition/test intervals produced partial or complete suppression of the test response, depending upon the location of the recording site, and upon the stimulus intensity. The inhibition could last more than 5 s; at brief intervals (50 ms), the test responses were shortened as a consequence of the enhanced inhibition arising from re-excitation of the mitral cells.

We conclude that, following a volley in the olfactory nerve, extremely prolonged changes in excitability occur. First, there appears to be a period of facilitation in the dendritic arborization of mitral cells. This is followed by a long lasting period of inhibition. The underlying neural mechanisms are unknown. However, the study of electrical phenomena at the level of the dendritic processes of neurons, particularly the spatio-temporal distribution of graded potentials, could be extremely important in elucidating the mechanisms of plasticity in this system as well as others.

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Initial survey of the chemosensory response properties of lobster mouthparts: spectral populations and tuning breadth. FRANK COROTTO AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

This is the first investigation of the physiological response properties of primary chemoreceptor cells from the third maxilliped of the lobster, *Homarus americanus*. Of interest were the presence of different spectral populations, and their tuning breadth, particularly in comparison with known cell populations in three other chemoreceptor organs in the same animal.

Chemoreceptor cells were located by injecting a one second search stimulus pulse (equimolar mixture of L-aspartate, L-glutamate, ammonium chloride, L-proline, sucrose, L-lysine, L-glutamine, betaine, L-arginine, hydroxy-L-proline, ethanol, L-alanine, glycine, taurine, and leucine), each at maximum applied concentrations of 10 μM into a carrier flow of artificial seawater which bathed the excised appendage. So far, 18 cells were located and tested with all 15 compounds injected separately as 1 s pulses with applied concentrations of 10 μM . Action potentials were recorded extracellularly.

Seven cells were narrowly tuned (responses to other compounds were minimal). Of these, three responded best to betaine, two to L-glutamate, one to ammonium, and one to taurine. Less narrowly tuned cells were also found: four responded best to hydroxy-L-proline, two responded best to glycine and one to L-arginine.

If receptor physiology reflects behavioral function, we speculate that taurine cells, common in antennules and thus far rare in maxillipeds, take advantage of the naturally high signal to noise ratio of taurine—due to its low background in seawater—to detect distant odor plumes. Maxillipeds guard food intake (Derby and Atema 1982, *J. Exp. Biol.* **98**: 317–327) which sets different constraints on the receptor cells of that taste organ. Our current survey indicates that maxillipeds are less specialized than antennules or walking legs in terms of receptor populations.

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Suppression of fictive feeding in vitro by foot shock in Limax maximus: neural correlates in withdrawal and feeding systems. K. DELANEY (Princeton University, Princeton, NJ 08544) AND J. J. CHANG.

Strong electric shock delivered to the head region produces a vigorous withdrawal response and cessation of feeding in intact *L. maximus*. This stimulus can be used to condition food aversion (Delaney and Gelperin 1984, *Neurosci. Abstr.* 10: 691). An *in vitro* preparation consisting of the central nervous system with lips attached to the cerebral ganglia generates a stereotyped bursting pattern in buccal ganglia nerves (fictive feeding, FF) when taste stimuli, such as potato juice, are applied to the lips. During a bout of FF, electrical stimulation of the anterior portion of the foot or the anterior pedal nerves (APN's) which innervate this tissue immediately stops all buccal nerve activity and bursting in buccal motor neurons recorded intracellularly. Thirty to sixty seconds of foot shock (0.5–5 Hz, 3–10 V) will block or reduce FF triggered with potato extract for up to 6 minutes after the shock. Suppression of FF is determined by comparing the number of bites in a feeding bout 2–10 minutes after foot shock with the mean number of bites in control bouts before and after the shock. Fifty percent of preparations showed suppression at 5 or 6 minutes post-shock ($n = 8$ preps showing suppression $> 25\%$ at 5 or 6 minutes, mean suppression 82%). No preps showed suppression at 10 minutes. Suppression of FF at all intervals was strongly correlated with transient suppression of spontaneous bursting in the salivary fast burster neuron suggesting that variability in suppression may relate to variability in the effectiveness of the shock stimulus.

Foot shock or APN stimulation produces inhibition of buccal motor neurons as determined by intracellular depolarizing current injection or decreases in spontaneous firing or bursting rates which persists for 30 seconds to 2 minutes. Strong inhibition of cerebral to buccal interneurons (CB's) which participate in triggering and modulating FF is reliably seen. CB₁, CB₃, CB₄, and CB_{EC} are inhibited for up to 6 minutes after foot shock and clearly show increased levels of spontaneous inhibitory synaptic inputs for up to 8–10 minutes post-shock. Taste-evoked activity in these CB's is reduced for up to 5 minutes post-shock. Spontaneous activity recorded in the nerve innervating the buccal mass retractor muscle, the muscle which produces head withdrawal is elevated for up to 20 minutes following 30 seconds of foot shock.

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Electromyographic recording of classical conditioning of eye withdrawal in the green crab. RICHARD D. FEINMAN (SUNY Health Science Center at Brooklyn, Brooklyn, NY 11203), CHARLES I. ABRAMSON, AND ROBIN R. FORMAN.

The eye withdrawal reflex of the green crab, *Carcinus maenas*, is an anatomically and physiologically well characterized system with many simplifying features that make it attractive for the study of neuronal correlates of learning. The main retractor muscle, 19a, is innervated by two neurons: a fast one that mediates retraction and a slow one that maintains the retracted state. The retraction proceeds without proprioceptive feedback and overrides more complex movements of the eyes. Myographic activity was recorded from the eye, using 50 μ silver wires inserted into muscle 19a during a classical (Pavlovian) conditioning procedure. The unconditioned stimulus (US) was a 0.5-s puff of air to the eye that invariably caused retraction into the carapace. This was correlated, in the myograms, with a rapid burst of spikes due to the fast retractor neuron followed by tonic firing by the slow retractor. The conditioned stimulus (CS) was a 5-s mild vibration to the carapace which had no effect on observable behavior of naive animals. Pairing of the CS with the US led to the appearance of retraction of the eye, and a characteristic electromyographic (EMG) response during CS presentations. The major findings were that: (1) myograms can be used to record the progress of classical conditioning of the eye withdrawal reflex. (2) The conditioned EMG response is, in general, of the same nature as the unconditioned EMG response, but is frequently more robust. (3) Acquisition of EMG response during CS presentation in animals with immobilized eyes, and their subsequent behavior in extinction and re-acquisition, is similar to the pattern of behavioral changes in unoperated animals. This indicates that the open loop nature of the reflex is preserved in this learning situation.

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Organization of the vestibulo-ocular and vestibulo-spinal reflex pathways in the toadfish, Opsanus tau: anatomy and electrophysiology. R. KITCH, T. C. TRICAS, AND S. M. HIGHSTEIN (Marine Biological Laboratory).

Retrograde transport of horseradish peroxidase (HRP), electrophysiology, and the intraaxonal injection of HRP were used to study vestibular reflex pathways in anesthetized toadfish. Eighth nerve branches

were labeled with HRP and alternate sections reacted with TMB/DAB. Five contiguous vestibular nuclei are distinguished after McCormick (1982, *J. Morph.* 171: 159): from rostral to caudal these are the nucleus anterior octavus (AO), n. magnocellularis (Mg), n. tangentialis (T), n. descending octavus (DO), and n. posterior octavus (PO). The central projections of the three semicircular canals (SCC) overlap extensively. They cover the entire AO, T, and PO, and the lateral and ventral aspects of both Mg and DO. The saccular and lagenar projections to the AO and PO overlies those of the SCC; however they do not project to T, and in contrast to the SCC, project dorsally to DO and Mg. The utricular nerve projections cover the entire AO, T, and PO nuclei, the medial and ventral regions of Mg, and are seen diffusely throughout DO, overlying both saccular and SCC projections.

Four central pathways were identified by intraaxonal recordings in the brain during ipsilateral eighth nerve stimulation. One tract projects anteriorly in the medial longitudinal fasciculus (MLF) to the oculomotor nuclei with a mean latency of 0.8 ms. Three descending pathways were identified: (1) an ipsilateral MLF pathway (mean latency = 0.8 ms, range = 0.5–1.2 ms). Intraaxonal injection revealed that these axons arose from cell bodies in Mg and descended to give a terminal collateral in the midline medullary reticular formation and terminals in the cervical anterior horn. (2) Another group of axons were recorded lateral and deep to group 1 and may correspond to the lateral vestibulo-spinal tract. (3) Finally, the contralateral MLF also contained a group of axons monosynaptically activated.

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Detection of chemical contrast in hermit crabs. LESLIE SAMMON AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543).

Contrast detection is an important feature of sensory perception, but poorly understood in chemoreception. Yet, recent results seem to indicate that chemoreceptor cells in lobsters are designed to enhance contrast (Atema 1985, *Soc. Exp. Biol. Symp.*, 39: 387–423): they are narrowly tuned and adjust response functions to background concentrations of their best compound (self-adaptation). They cross-adapt to compounds that stimulate the cell.

We developed a behavioral assay to test chemical contrast detection in whole animals by measuring hermit crab (*Pagurus longicarpus*) responses to two complex odors against four different backgrounds. In a small flume, six animals were presented for 5 minutes with a stimulus concentration series of fish extract (FE) and mussel extract (ME) injected into a background of raw seawater (RSW). A response function resulted ($n = 24$ animals for each dilution). FE was slightly more stimulatory than ME. When RSW backgrounds were changed to 10^{-4} FE or ME backgrounds, the response functions shifted up to the level of the background indicating self-adaptation (ME in ME or FE in FE), but were unaffected in cross-adaptation (ME in FE or FE in ME). The responses to ME and FE dropped in artificial seawater (ASW) background. This contradicts our expectation that lower organic backgrounds would result in greater contrast and enhanced responses.

We then tested effects of living in an altered organic background by keeping the crabs for 7 days in ASW, or in 10^{-4} dilution of FE or ME. ASW-exposed animals responded as in RSW, apparently adjusting to this background that had suppressed their responses in the short term; responses were still not enhanced as predicted. FE and ME-exposed animals showed response functions similar to those in short term self- and cross-adaptation. When tested subsequently in RSW, the response remained suppressed indicating that changes in thresholds may persist after long term exposure to high background levels.

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Migratory behavior of individual horseshoe crabs. MARY ANNE SYDLIK, ROBERT B. BARLOW JR., ANDREW STULL, DONALD R. NELSON, AND CARRIE KAMMIRE (Marine Biological Laboratory).

During the breeding season horseshoe crabs (*Limulus polyphemus*) migrate to shore for mating and nesting. On Cape Cod these migrations are correlated with lunar cycle, tidal cycle, and time of day. During the 1987 season we tracked the migrations of individual males and females with tags and a computerized ultrasonic telemetry system.

We tagged 576 individuals (152 clasped pairs, 253 solitary males, and 19 unmated females) over a four-week period. We found that animals were most likely to revisit the beach within two to three days of tagging. This suggests that individuals within the population have different rhythms for when to move into shore or that they use the same beach for a few days and then switch to another location. There was no relationship between the time of day an animal was tagged and when it was next seen at the beach. A larger percentage of unmated males (24%) than individuals tagged as part of a pair (12%) were found on the beach

on subsequent tides. Fewer females (8%) than males (18%, solitary and clasped combined) were seen at the beach on more than one occasion, indicating that males and females may use different rules for how often to travel into shore and therefore may respond differently to environmental cues for migration.

An unmated male, an unmated female, and a clasped female were fitted with ultrasonic transmitters. All three animals were subsequently located in the bay using hand held hydrophones. We used triangulated hydrophones and a computer to track the path of the male as he moved within the mating area.

We conclude that tagging and computerized ultrasonic tracking, combined with measures of environmental variables at the staging site, can provide a rich source of information about the factors influencing movement patterns of individual horseshoe crabs.

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NEUROBIOLOGY

Characterization of phospholipid enzymes in squid axoplasm and giant fiber lobe.

MARIO ALBERGHINA, SERAFINA SALVATI, AND ROBERT GOULD (Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY).

Proteins and phospholipids synthesized in the neuronal perikarya are axonally transported at a rapid rate as part of the mechanism for membrane growth and maintenance in the axon and synaptic endings. In addition, phospholipid enzymes are transported to axons and synaptic endings where they can modify the structure of membranes in these specialized neuronal regions. The squid giant axon is an ideal preparation to demonstrate lipid enzymes in axoplasm since pure axoplasm can be obtained by simple extrusion and axoplasmic enzymes can be characterized biochemically. Here we report on the characteristics of three axonally localized enzymes, (1) CDP-diacylglycerol:myo-inositol phosphotransferase, (2) acyl-CoA:1-acyl-sn-glycero-3-phosphocholine acyltransferase, and (3) phospholipase A₂. Activities in axoplasm were compared with activities in the neuronal perikarya-rich giant fiber lobe (GFL), and other neural tissues of the squid, *Loligo pealei*. Properties of phosphatidylinositol synthase (enzyme 1) were quite similar for axoplasm and giant fiber lobe extract. Km values were: 0.44 mM (axoplasm) and 0.8 mM (GFL) for myo-inositol and 0.025 mM (axoplasm) and 0.030 mM (GFL) for CDP-dicaproin. Both activities were activated by Mg⁺⁺, inhibited by low (0.01 mM) concentrations of Ca⁺⁺ and Mn⁺⁺, and were inactivated by heating (70°C for 10 min). One striking difference was found: the axoplasmic activity was strongly inhibited by N-ethylmaleimide whereas the GFL enzyme was rather insensitive to this sulfhydryl group reagent. Acyltransferase activity in axoplasm, GFL, optic nerve, optic lobe, and fin nerve had similar properties, including: Km values for lysophosphatidylcholine [*i.e.*, 0.008 mM (axoplasm) and 0.01 mM (GFL)] and for oleoyl-CoA [*i.e.*, 0.003 mM (axoplasm) and 0.005 mM (GFL)], pH optimum (7.4–8.0), inactivation by heat and by sulfhydryl group reagents. Phospholipase A₂ activity was characterized with 1-palmitoyl-2-[¹⁴C]-oleoyl-sn-glycero-3-phosphocholine as substrate. In all tissues examined, enzyme activity was linear with protein concentration and time for up to two hours. In axoplasm and GFL, activity exhibited a pH optimum between 7.4 and 8.0. The activity was sensitive to heat and p-bromo-phenylacetyl bromide. Calcium was not required for full activity. These results further characterize the *de novo* synthesis of phosphatidylinositol in axoplasm and demonstrate that there are enzyme activities in axoplasm that modify the acyl side chains of phospholipids through the acylation-deacylation cycle.

Supported by a grant from the NIH (NS 13980, RMG).

Calcium block of K channels in squid giant fiber lobe neurons. C. M. ARMSTRONG (University of Pennsylvania, Philadelphia, PA) AND Y. PALTÍ.

We studied the block of K channels by internal calcium and strontium ion in cells isolated from the giant fiber lobe of the squid stellate ganglion. Freshly dispersed cells have large potassium currents, a small calcium current, and essentially no sodium current. Cells were whole-cell patch-clamped, using pipettes that contained 20 mM CaCl₂ or SrCl₂, plus KCl and K glutamate. The external solution was 440 mM NaCl and 50 CaCl₂. The Ca or Sr effect developed with a lag, making it possible to record near-normal K current immediately after breaking into the cell. Ca and Sr blocks were apparent only at membrane voltages above about 50 mV. Below this level K current had an almost normal appearance. Above 50 mV, current developed normally for a fraction of a millisecond, and then "inactivated" as Ca or Sr entered the channel. Block deepened with increasing voltage up to about 150 mV, but was never complete, with about 15% of current remaining. Calcium entry rate could be measured by opening the channels at 30 mV, where block is slight, then following the decline of the current following a step to higher voltage. On stepping from 30 to 130 mV, current jumped in magnitude by a factor of about two because of increased driving force, then

decayed to about one-third of this level with a time constant of about 0.5 ms. Analysis suggests that the calcium entry rate increases with voltage. Calcium-blocked channels at 130 mV recover from block when voltage is lowered to 30 mV, with a time course that approximates the final phase of channel opening on stepping from -70 to 30 mV. The results in general are compatible with the idea that calcium block is an element of normal gating, and that the final step in opening of a K channel involves the release of a calcium ion bound in the channel. Driving calcium into the channel from inside appears to reverse this final step.

Adenosine depresses spontaneous transmitter release from frog motor nerve terminals by acting at an A1 receptor. SUSAN R. BARRY (University of Michigan).

During nerve stimulation adenosine triphosphate (ATP) may be released with acetylcholine from vertebrate motoneurons and hydrolyzed to adenosine in the synaptic cleft (Silinsky 1975, *J. Physiol.* **247**: 145-162). Since adenosine levels at the neuromuscular junction (NMJ) may increase with neuronal activity, adenosine may play a feedback role in neuromuscular transmission.

Adenosine depresses spontaneous transmitter release from frog motor nerve terminals. Spontaneous miniature endplate potentials (mepps) were recorded with intracellular microelectrodes from motor endplates of the frog cutaneous pectoris muscle. Adenosine reversibly depressed mepp frequency by up to 50% but had no effect on mepp size. This inhibitory action was mediated by an adenosine receptor since the effect of 10 μ M adenosine was blocked by 20 μ M theophylline, an adenosine receptor antagonist.

Two types of adenosine receptors, A1 and A2, have been described on several cell types. Adenosine depresses adenylate cyclase activity at the A1 receptor but enhances adenylate cyclase activity at the A2 receptor. Adenosine analogs act at nanomolar concentrations at the A1 receptor but at micromolar concentrations at the A2 receptor. The analog, L-N⁶-phenylisopropyladenosine (L-PIA), is more potent than 5'-N-ethylcarboxamidoadenosine (NECA) at the A1 receptor, while the reverse is true at the A2 receptor. Finally, L-PIA is at least 100 times more potent than its stereoisomer, D-PIA, at the A1 receptor but only three times more potent than D-PIA at the A2 receptor.

The effects of L-PIA, NECA, and D-PIA were tested on spontaneous transmitter release at the frog NMJ. All three compounds depressed mepp frequency without affecting mepp size. The threshold concentration for the effect of L-PIA was about 1 nM. L-PIA was 12 times more potent than NECA and 300 times more potent than D-PIA at reducing mepp frequency. These data indicate that adenosine depresses spontaneous transmitter release from frog motoneurons by acting at an A1 receptor. Future experiments will determine whether adenosine reduces spontaneous transmitter output by inhibition of adenylate cyclase.

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Do arachidonic acid metabolites mediate modulation of K and Ca currents by FMRFamide in Aplysia neurons? VLADIMIR BREZINA (Department of Biology, UCLA).

Piomelli *et al.* (1987, *Nature* **328**: 38-43) recently suggested that lipoxygenase metabolites of arachidonic acid act as second messengers mediating the inhibitory response, due to increased activity of K channels of the 'S' type, to the neuropeptide FMRFamide in *Aplysia* sensory neurons. Work in our laboratory (Brezina *et al.* 1987, *J. Physiol.* **382**: 267-290; **388**: 565-595) showed that the inhibitory response to FMRFamide in other identified *Aplysia* neurons, the abdominal ganglion cells L2-L6 and R2, also consists of enhancement of the 'S' or a similar K current (as well as suppression of the Ca current). Therefore I used the methods of Piomelli *et al.* to investigate if the FMRFamide response in these cells might also be mediated by arachidonic acid metabolites.

In the sensory neurons, Piomelli *et al.* found that the effect of FMRFamide was blocked by 4-bromophenacyl bromide, an inhibitor of receptor-mediated release of arachidonic acid from membrane phospholipids, as well as by nordihydroguaiaretic acid, which inhibits lipoxygenase pathways of arachidonic acid metabolism, but not by indomethacin, an inhibitor of the cyclooxygenase pathway. In voltage-clamped L2-L6 and R2 cells, none of these inhibitors (up to 100 μ M) had any effect on the FMRFamide response. Piomelli *et al.* also found that direct application to sensory neurons of arachidonic acid (dissolved in toluene, then dried and redissolved in the bath medium) mimicked the effect of FMRFamide. In cells L2-L6 and R2, this procedure, like FMRFamide, also elicited large currents that were outward at -40 mV and were probably carried by K⁺. However, further investigation showed that the active substance was not arachidonic acid, but contaminant toluene. Low concentrations ($\leq 0.1\%$) of toluene alone (as well as of benzene, xylene, and hexane, another solvent used by Piomelli *et al.*) elicited currents with current-voltage characteristics identical to those presented by Piomelli *et al.* It also reproduced the hyperpolarization of sensory neurons that Piomelli *et al.* attributed to the action of arachidonic acid.

I conclude that there is no present evidence to suggest that the effect of FMRFamide on cells L2-L6 and R2 is mediated by arachidonic acid metabolites. Furthermore, many of the experiments of Piomelli *et al.* demonstrate effects of the solvent, not of arachidonic acid.

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The effect of strontium, barium, and strychnine on the synapse made by barnacle photoreceptors. JOSEPH C. CALLAWAY AND ANN E. STUART (University of North Carolina).

The presynaptic terminals of barnacle (*Balanus nubilus*) photoreceptors (PRs) have a K conductance activated by the entry of Ca (CaGK). This CaGK might affect the membrane potential locally at the release sites, influencing the release of transmitter and thus the postsynaptic (I-cell's) response. We tested this idea by attempting to eliminate the CaGK while recording the I-cell's response to a 2 s pulse of light. Normally this response consists of an initial peak that sags to a plateau. We tested whether substitution of Ca with Sr or Ba might eliminate the PR's CaGK and eliminate this sag.

Twenty millimolar Ba (0 Ca) permeates the cell but does not activate the CaGK. It causes an action potential (AP) without the prolonged undershoot which is due to the CaGK. However, in 20 mM Ba the I-cell's response is totally abolished. Two millimolar Ba added to a normal (20 Ca) saline does not eliminate the AP undershoot in the PR and thus does not block CaGK from the outside. We suspect that no Ba enters the cell due to the much stronger affinity of Ca for an intrapore binding site (Tsien *et al.* 1987, *Ann. Rev. Biophys. Chem.* **16**: 265–290). 2 mM Ca added to 20 mM Ba decreases the duration of the AP, as if it were preventing Ba entry.

Twenty millimolar Sr (0 Ca) supports release of transmitter but also activates the CaGK, since the AP in the PR has an undershoot in the Sr saline. Thus neither Ba nor Sr substitution have proven useful in testing the hypothesis.

Surprisingly, strychnine (10^{-4} M) eliminates the sag in the I-cell's light response. Strychnine, however, does not affect the presynaptic CaGK. It has no effect on the light response recorded in the PR terminals or on the amplitude, duration, or undershoot of APs set up there (in 20 mM Ca and 20 mM tetraethylammonium ion). This suggests that the sag is a postsynaptic phenomenon and is not due to the PR's CaGK.

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Resting conductance of the squid axon membrane. D. C. CHANG, J. R. HUNT, AND P. Q. GAO (Baylor College of Medicine).

The resting membrane of the nerve cell is semi-permeable to cations. Our previous studies suggest that such permeability properties are not determined by the excitable Na and K channels which are responsible for generating the action potential. To characterize the conductance pathways of the resting membrane, we employed internal perfusion and voltage clamp techniques to study the current-voltage relationship of squid giant axons near the resting membrane potential. Tetrodotoxin was used to block the excitable Na channel, and tetraethylammonium (TEA) and/or 4-aminopyridine (4-AP) to block excitable K channels. In most experiments symmetrical internal and external K concentrations (100 mM) were used to shift the K⁺ reversal potential away from the resting potential. Under these conditions, we found a small component of K conductance (denoted here as g_{K2}) which is activated at a potential far more negative than the classical delayed rectifier K channel (g_{K1}). With a long depolarizing pulse (100 ms) the I-V curve of steady-state current shows two "hooks," one of which represents inactivation of g_{K2} and the other inactivation of g_{K1} . While g_{K2} is not blocked by 20 mM TEA, it appears to be blocked by 2 mM 4-AP. Its ion selectivity is K > Rb > NH₄ > Na. At this point it is not clear whether g_{K2} is completely unrelated to g_{K1} or is a component of g_{K1} induced by high [K]_o. When both TEA and 4-AP are applied, both g_{K1} , and g_{K2} are removed, but the resting membrane potential is reduced by only 10 mV, and the resting potential is still strongly dependent on [K]_o, but is not very sensitive to [Na]_o, much like an untreated axon. Furthermore, the resting conductance under TEA + 4-AP still shows an ion selectivity of K > Rb > NH₄ > Na. These observations suggest an additional K conductance, g_{K3} , contributes to the resting potential. We find that g_{K3} is not measurably voltage dependent. In conclusion, our results suggest multiple pathways (e.g., g_{K2} and g_{K3}) for the resting current in the squid giant axon.

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Classification of presynaptic calcium channels in the squid giant synapse. MILTON P. CHARLTON (University of Toronto) AND GEORGE J. AUGUSTINE.

Recent studies demonstrate that pharmacological and other criteria may be used to classify Ca channels into at least three different types. We attempted to extend this classification scheme to the Ca channels responsible for mediating transmitter release at the squid giant synapse.

Our experiments focused on two different types of ligands—omega toxin from the snail *Conus geogra-*

phus and dihydropyridines—that were used to discriminate between the two types of Ca channels proposed to mediate transmitter release at other synapses. These agents were dissolved in saline containing 1/5 of the normal Ca concentration and were perfused through the artery that normally supplies blood to the squid stellate ganglion. In this saline, the giant synapse responds to presynaptic action potentials with subthreshold synaptic responses (PSPs) that we used as indirect, but sensitive, indicators of the presynaptic Ca channel activity.

Omega Conus toxin had no effect on PSPs at concentrations as high as 500 μ M. Addition of cytochrome C (1 mg/ml) to the saline to reduce nonspecific toxin binding did not increase the effectiveness of the toxin. Reduction of the divalent ion concentration of the saline to 0.75 mM Ca and 15.5 mM Mg still did not allow the toxin to decrease PSP amplitude. Further, the dihydropyridine Ca channel antagonist nitrendipine (1–10 μ M) and the Ca channel agonist Bay K 8644 (1–10 μ M) had no effect on PSP amplitude.

In summary, neither Conus toxin nor dihydropyridines alter transmission at the squid giant synapse. From this we conclude that there must be more than three types of Ca channels because the Ca channels responsible for mediating transmitter release at this synapse do not correspond to any of the three types of Ca channels previously classified.

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C-kinase activation mediated by proteolysis modulates K⁺ conductances in Hermisenda B-photoreceptors. CHONG CHEN (Caltech 164-30, Pasadena, CA 91125), DANIEL L. ALKON, AND PAUL E. GALLANT.

C-kinase activation by phorbol esters together with Ca²⁺-loading conditions reduces *Hermisenda* K⁺ currents. I_A and I_C and enhances I_{Ca}²⁺ (Alkon *et al.* 1986, *Biochem. Biophys. Res. Comm.* **134**: 1245–1253; Farley and Auerbach 1986, *Nature* **319**: 220–223). The activation depends on translocation of C-kinase from the cytosolic to the membrane compartment. It was initially reported that K-kinase could be activated by limited proteolysis with trypsin or calpain, forming an enzymatically active M-kinase (Inoué *et al.* 1977, *J. Biol. Chem.* **252**: 7610–7616). The physiological significance of the activation of C-kinase by protease remains obscure. The two-microelectrode voltage-clamp technique was used to study whether the proteolysis of C-kinase could modulate membrane conductances of isolated B-photoreceptor somata. The cell was held at –60 mV. A +60 mV voltage step elicited two major K⁺ currents, I_A and I_C. The average currents before addition of the drug were 50 ± 5.5 nA S.E. for I_A and 22.0 ± 2.5 nA for I_C. The iontophoretic injection of trypsin inhibitor, leupeptin, 25 μ g/ μ l in 0.9 M KAC reduced I_A to 86.7 ± 2.5% S.E. (n = 18) and I_C by 88.8 ± 2% S.E. (P < 0.005). Pre-exposing cells to 0.5 μ M phorbol ester DPBA reduced the effect of leupeptin injection on the I_A to 97.8 ± 2.7% S.E. and to a lesser extent I_C to 91.7 ± 4%. Bath application of C-kinase inhibitor H₇ (60 μ M in ASW) reduced the effect of leupeptin injection on I_A to 95.9 ± 3.0% and I_C to 97.7 ± 4%. The iontophoresis of trypsin, 2.5 μ g/ μ l in 0.9 M KAC, increased I_A to 106 ± 3% and I_C to 107 ± 2%. The DPBA exposure reduced the leupeptin effect on I_A to 99.7 ± 1.5% and on I_C to 100.1 ± 1.6% of control. This study demonstrates an interaction between protease inhibitor/stimulator and C-kinase inhibitor/stimulator. The study suggests that there may be another C-kinase activation pathway which also modulates K⁺ channels. The C-kinase activation by proteolysis interacts with phorbol esters/Ca²⁺-activated C-kinase in such a way that they modulate K⁺ channels in two opposite directions, i.e., enhancement and reduction.

Inactivation rate is not voltage dependent in pituitary sodium channels. GABRIEL COTA AND CLAY M. ARMSTRONG (Dept. of Physiology, University of Pennsylvania).

Papain inside GH3 cells removes the inactivation of the sodium channels. We recorded macroscopic sodium currents at 15–20°C with patch pipettes, using supercharging to increase voltage-clamp speed (Armstrong and Chow 1987, *Biophys. J.* **52**: 133–136). Papain (1 mg/ml) was delivered through the patch pipette. The sodium current usually changed little within the first three minutes of whole-cell recording, and then gradually increased in size with a clear prolongation of its rising phase and loss of inactivation (incomplete decay of the current during the 10-ms test pulse). The enzymatic removal of fast inactivation was nearly complete 10 min later. As in neuroblastoma cells (Gonoi and Hille 1987, *J. Gen. Physiol.* **89**: 253–274), the prolongation of the rising phase and the increase of current amplitude induced by papain were more pronounced for a small depolarization than for a larger one.

The rate constant of the inactivation step can be determined by comparing current traces at several voltages before and after removal of inactivation. The rate constants determined at –20, 0, +40, and +60 mV were 1.53, 1.58, 1.44, and 1.50 ms^{–1}, respectively (15°C, 1 mM external Ca). We conclude that sodium channels inactivate with a voltage-independent rate constant.

Incorporation of tritiated inositol and choline into phospholipids in the squid stellate ganglia with special reference to the giant synapse. ROBERT M. GOULD (Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY), JOHN HOLSHEK, AND DAVID W. PUMPLIN.

Isolated squid stellate ganglia with attached pallial nerves and hindmost giant axons were incubated in artificial seawater (MBL formulation) at room temperature (22°C) with either tritiated inositol or tritiated choline (200 μ Ci/3 ml) for three hours. The preparations were washed in seawater and some were stimulated for 10 minutes at 10 Hz. They were then fixed in 2.5% glutaraldehyde in 0.8 M sucrose and 0.1 M cacodylate buffer, pH 7.4, for several hours. After fixation, the samples were washed for several days with buffer to remove water soluble precursors, embedded in Spurr's embedding resin under conditions that limit extraction of the lipid. Light and electron microscopic (EM) autoradiographs were then prepared (Gould *et al.* 1987, *J. Neurochem.* 48: 1121–1131). With light microscopic autoradiography, the major sites of phosphatidylcholine (choline precursor) and phosphatidylinositol (inositol precursor) formation were the cell body-rich giant fiber lobe and the neuropil adjacent to the giant fibers and their synaptic specializations. Choline appeared to label the neuronal perikarya more strongly than inositol. Labeling at the synapse was higher than in the pre- or postsynaptic axoplasm. Quantitative EM autoradiography was used to characterize the relative incorporation by different pre- and postsynaptic elements. Both choline and inositol were selectively incorporated into lipid within glial elements at the junction. One difference between the precursors was that lipid formation from choline was more prominent in postsynaptic structures and in postsynaptic axoplasm, whereas lipid formation from inositol showed preference for presynaptic structures including the regions rich in synaptic vesicles. A further study compared inositol lipid distribution between a resting preparation and one stimulated (10 min at 10 Hz) following labeling. We found that the stimulated preparation showed a marked decline in the proportion of silver grains over postsynaptic structures compared with the non-stimulated preparation. Further preparations are being analyzed to substantiate these findings.

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Two classes of miniature end-plate potentials are present in the isolated, innervated electrocyte. M. E. KRIEBEL (Dept. Physiology, Health Sci. Ctr., Syracuse, NY), G. D. PAPPAS, AND G. Q. FOX

The electric organs of the skate (*Raja erinacea*) were treated with 1% collagenase in elasmobranch saline for two hours so that individual electrocytes could be separated with gentle shaking. These electrocytes were washed with saline and placed in a small bath attached to the stage of a compound microscope. Some small electrocytes were spherical (50 μ m dia.) and innervated on about 30% of their surface. The innervated surface invaginates during differentiation to form the "cup" shape of the mature cell. Intracellular recordings from electrocytes show resting potentials of -65 mV. Nerves were stimulated by drawing the innervated surface against a suction electrode and threshold analyses indicate three to six efferent nerves. End-plate potentials (50 mV) and spontaneous intracellular miniature end plate potentials (MEPPs) were recorded. The two classes of MEPPs from small cells are based on amplitudes. The smaller class (sub-MEPPs) is about $1/10^{\text{th}}$ the size of the larger class (1–2 mV). The smallest electrocytes show the greatest percentage of sub-MEPPs. Both classes have the same time characteristics which indicate that both classes are generated by the same postsynaptic mechanism. Both classes are recorded at the same site showing that they are from the same presynaptic release area. If vesicle size alone determines quantal size then there should be two classes of vesicles based on diameters (with volume ratios of 10:1). Histograms of synaptic vesicle diameters show a smooth, slightly log-normal distribution with a mean of 60 nm. No vesicles with a mean of 30 nm were found to account for the sub-MEPP quantal class.

Autoradiography utilizing [^3H] phorbol esters is potentially useful for cellular analysis of protein kinase C activity in hippocampus slices. ALAN M. KUZIRIAN (Lab. of Biophysics, NINCDS-NIH, Marine Biological Laboratory), BARRY BANK, JOSEPH LOTURCO, AND DANIEL L. ALKON.

Recent studies characterizing eyelid conditioning induced changes in ionic currents in the CA1 pyramidal cells of rabbit hippocampus implicate protein kinase C (PKC) (Disterhoft *et al.* 1986, *PNAS* 83: 2733–2737). The current changes resemble those previously reported for *Hermisenda* B photoreceptors following conditioning and direct iontophoretic injection of purified PKC (Kubota *et al.* 1986, *Soc. Neurosci. Abstr.* 12: 599). Bank *et al.* (manuscript in review) found evidence for classical conditioning (eyelid)

induced translocation of cytosolic PKC into the membranes of CA1 cells but with no change in total activity.

Because these conditioning specific changes are similar in both animals and are mimicked by phorbol esters which specifically bind to PKC, we attempted to determine the distribution of PKC at the cellular level using [^3H]PDBu in rabbit hippocampus (CA1 region).

Hippocampus slices (300–700 μm thick) from conditioned, pseudoconditioned, and naive animals were generally prefixed (2.5% glutaraldehyde in 0.05 *M* cacodylate, pH 7.4; 10 min), incubated in either 2.5 nM [^3H]PDBu alone or combined with 2.5 μM non-radioactive PDBu (non-specific labelling control) for 60 min, buffer washed (30 min), further fixed (60 min), and embedded in 5% agarose. Vibratome sections (10–20 μm) were cut and mounted on subbed slides, coated with Ilford KD-5 emulsion, exposed for 4 to 6 weeks, and developed. Silver grains were counted over the strata oriens and radiatum and pyramidal cell layer.

Scintillation data of treated sections indicated no binding activity differences between prefixed or fresh slices while non-specific labelling represented approximately 50% of the total amount bound under both conditions. Silver grains were equally distributed over the strata oriens and radiatum as well as CA1 soma region (4.7 ± 0.2 grains/100 μm^2). Silver grain counts from slices incubated with [^3H] and non-radioactive PDBu decreased slightly over the CA1 soma region and stratum oriens (4.2 ± 0.1 grains/100 μm^2). However, the number of silver grains decreased 49% over the stratum radiatum (2.3 grains/100 μm^2).

These results compliment those of Worley *et al.* (1986, *J. Neurosci.* 6: 199–207). However, data from the non-specific binding experiment clearly show that PKC is concentrated in the stratum radiatum (the area containing the CA1 apical dendrites) with a minor amount in the stratum oriens adjacent to the CA1 soma region. Although no statistically significant differences in silver grain densities over the areas sampled could be directly related to training differences, this possibility will be pursued.

Calcium affects the birefringence response of the squid giant axon. DAVID LANDOWNE (University of Miami School of Medicine).

Segments of squid axons were internally perfused and voltage-clamped with an axial internal electrode assembly. A beam of plane polarized light from a tungsten-halogen bulb passed through the preparation from below at 45° with respect to the axis. A second polarizer perpendicular to the first was mounted above the nerve and a photodetector placed above this analyzer. The birefringence response is seen as a change in light level at the detector associated with a change in nerve membrane potential. The control external solution contained (in mM) 88 Na, 352 TMA (tetramethylammonium), 2 HEPES buffer and 50 Ca.

During a depolarizing voltage pulse the birefringence decreases rapidly for the first few hundred microseconds and then more slowly for the first few milliseconds. After the pulse the birefringence returns to the resting level at first rapidly and then more slowly. The sodium permeability of the membrane increases with a delay after the fast decrease in birefringence. At the end of a brief pulse the permeability decreases with the same time course as the rapid birefringence change. These two changes in birefringence, the rapid and the slower change, have been associated with molecular motion underlying activation and inactivation of the sodium channels respectively (Landowne, 1985, *J. Membr. Biol.* 88: 173–185).

Elevating the external Ca concentration from 50 mM to 250 mM at constant Na, while maintaining osmolarity by replacing TMA, decreases the amplitude and rate of rise of the sodium permeability change during the pulse and increases the rate of decline following the pulse. High Ca decreases the slope of the slow birefringence change both during and after the pulse. The rapid change after the pulse is distinctly more rapid in high Ca, the rapid change at the beginning of the pulse is unchanged or slightly faster. Reducing Ca to 10 mM (TMA replacement) has the opposite effects, increasing the slope of the slow changes in birefringence and slowing the fast recovery after the pulse. These results illuminate the molecular basis of calcium's effect on the sodium permeability change and thus on nerve excitability.

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Sectionless sectioning: a systematic method for scanning electron microscopic examination of embedded tissue. STEPHEN B. LEIGHTON AND ALAN M. KUZIRIAN (NINCDS-NIH, Marine Biological Laboratory, Woods Hole, MA 02543).

We have developed a new method for obtaining serial images of epoxy embedded tissue, without much of the tedium, distortion, and possible loss of sections associated with traditional sectioning. A miniature microtome was built which operates inside a scanning electron microscope (SEM). As sections are cut, they are discarded and the remaining block face is sequentially: etched with an oxygen plasma, coated by a gold sputtering device, and imaged—all within the SEM. Because the block face is imaged while fixed with respect to the electron optics (except to advance for the next cut), problems of section handling,

distortion, and alignment are avoided. The plasma etching differentially etches the epoxy faster than the tissue, thus providing relief to the block face and improving image quality.

Serial images were obtained of a nerve trunk emanating from the pedal ganglion of *Hermissenda crassicornis*, which was fixed in glutaraldehyde/osmium and embedded in epoxy resins. Section thickness was approximately 0.75 micron and features as small as 100 nm were resolved. Use of a tungsten filament in the SEM reduced the beam current to a low enough level (300 picoamps at 10 kV) to eliminate the problem of beam damage to the block, previously observed with a Lanthanum Hexaboride filament.

We anticipate that this technique may significantly aid three-dimensional reconstructions of a broad range of biological tissues including neural networks and whole embryos for ontogenetic studies. It may also be useful for systematic sampling of tissues in the SEM, concurrent with x-ray analyses. The method uses to its best advantage the large depth of field and full range of magnifications of the SEM to sample large surface areas rapidly, thus making it ideal for bridging the gap between light microscopy and TEM. The system also has the potential to be automated to a high degree.

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Behavioral experiments suggest G protein modulation of calcium channels in Paramecium. ANITA D. MCILVEEN (University of Connecticut Health Center, Farmington, CT), SARAH GARBER, AND BARBARA E. EHRLICH.

Backward swimming of *Paramecium* is regulated by the opening of voltage-dependent calcium (Ca) channels in the cilia. Previous work showed that the duration of backward swimming in potassium-containing solutions correlates with the magnitude of the Ca current. Using this behavioral assay, we tested compounds that may modulate Ca channels in *Paramecium calkinsi*, a marine ciliate. To incorporate putative regulatory compounds intracellularly, cells were permeabilized with EDTA (1 mM). After addition of the test compound(s), the cells were resealed with 1.2 mM MgCl₂. In all cases 6-carboxyfluorescein was added as a permeabilization indicator. After washing the cells, the effect of the test compound(s) on the behavior of fluorescent cells was measured. We found that intracellular concentrations of GTP γ S, a nonhydrolyzable analog of GTP which binds to and activates G proteins, of 1 μ M and greater caused the cells to swim backwards irreversibly (≥ 900 seconds). Mean recovery time for control cells was 180 seconds. Other compounds such as GDP β S, ATP γ S, AMPPNP, and GNPPNP (each at 100 μ M) had no effect on the length of backward swimming suggesting that the action of GTP γ S is specific. When 1 μ M GTP γ S and 10 μ M GDP β S were incorporated simultaneously, the effect of GTP γ S alone was entirely eliminated. These results strongly suggest that the backward swimming behavior of *Paramecium* is modulated by G proteins. Since behavioral experiments cannot distinguish between an effect on the Ca channel and an effect on the switch controlling the direction of ciliary motion, electrophysiological experiments will be used to distinguish between these alternatives.

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Voltage-clamp reversal of the sodium pump in dialyzed squid giant axons. R. F. RAKOWSKI, DAVID C. GADSBY, AND PAUL DE WEER (Marine Biological Laboratory).

We have used a low-noise voltage clamp to examine current-voltage (I-V) relationships of the electrogenic sodium pump in internally dialyzed squid giant axons under various experimental conditions. Na and K conductances were inhibited by 200 nM tetrodotoxin and 1 mM 3,4-diaminopyridine in the bathing solution and 20 mM 3-phenylpropyltriethylammonium in the dialysate. The I-V relationship of the Na pump was determined as the difference between steady-state I-V curves obtained in the presence and absence of 0.1 mM dihydrodigitoxigenin (H₂DTG). Membrane currents in the presence of H₂DTG were insensitive to external [K]. Under conditions designed to support Na/K pumping in the forward direction only (50 mM Na_i, 0 Na_o, 0 K_i, 10 K_o, 5 ATP), pump current declines slightly and linearly with hyperpolarization (10–20% between 0 and –60 mV). Addition of 400 mM external Na renders the pump current more steeply voltage dependent (30–50% decline between 0 and –60 mV), with a hint of nonlinearity at very negative potentials (< –100 mV). When the sodium pump is constrained to run backward only (0 Na_i, 400 Na_o, 140 K_i, 0 K_o, 5 ATP, 5 ADP, 5 P_i), its I-V relationship is sigmoidal, saturating at voltages more negative than –100 mV and approaching zero at positive potentials (10 μ M atractyloside and 2 mM diadenosine pentaphosphate were present to suppress endogenous ATP generation.) We also produced conditions in which both forward and reverse pumping can occur within the accessible range of membrane

potentials, by lowering dialysate [ATP] to 0.1 mM and raising [ADP] and [P_i] to 10 mM each. Resulting pump I-V relationships were approximately linear from -100 to +20 mV, and clearly crossed the zero current axis. Our observations demonstrate that the direction of the sodium pump under constant ionic and biochemical conditions can be reversed by varying membrane potential alone. The pump remains electrogenic on either side of reversal without abrupt change in slope, allowing determination of pump reversal potential by interpolation.

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Calcium channels required for neuropeptide release in the intact nerve terminals of vertebrate neurohypophyses are sensitive to ω -conotoxin and insensitive to dihydropyridines: optical studies with and without voltage-sensitive dyes. B. M. SALZBERG, A. L. OBAID, AND R. FLORES (University of Pennsylvania).

Optical techniques can facilitate the study of excitation-secretion coupling, especially in the small nerve terminals of vertebrates. Extrinsic absorption changes exhibited by potentiometric dyes have established the ionic basis of the action potential in synchronously activated populations of nerve terminals in the intact amphibian and mammalian neurohypophyses (Salzberg *et al.* 1983, *Nature* **306**: 36-40; Obaid *et al.* 1985, *J. Gen. Physiol.* **85**: 481-489.) Also, large and rapid changes in light scattering, measured as transparency, follow membrane depolarization and are intimately associated with the release of neuropeptides from the nerve terminals of the mouse neurohypophysis (Salzberg *et al.* 1985, *J. Gen. Physiol.* **86**: 395-411; Gainer *et al.* 1986, *Neuroendocrinology* **43**: 557-563).

We report some experiments that help to define the pharmacological profile of the calcium channels present in intact neurosecretory terminals of vertebrates. We used the dihydropyridine compounds nifedipine and Bay-K 8644, at concentrations ranging from 2 to 5 μ M, and the peptide toxin ω -conotoxin GVIA (Peninsula) (1-5 μ M). In the frog neurohypophysis, neither dihydropyridine compound had any effect on the calcium-dependent components of the action potential or on the regenerative calcium response elicited in the presence of 1-2 μ M TTX/5 mM TEA. These spikes remained absolutely unchanged for up to one hour in the presence of nifedipine and Bay-K 8644. ω -Conotoxin GVIA, however, abolished the after-hyperpolarization of the normal action potential, and dramatically reduced the height of the upstroke and the size of the undershoot in the regenerative calcium spike.

The effects of these calcium channel modifiers on the action potential recorded optically from the terminals of the *Xenopus* neurohypophysis was faithfully reflected in the behavior of the light scattering changes observed in the neurohypophysis of the CD-1 mouse. Dihydropyridines left unaffected the intrinsic optical signal associated with secretion, while ω -conotoxin GVIA (5 μ M) reduced its size by 50%. These observations demonstrate for the first time that the type of calcium channels that dominates the secretory behavior of intact vertebrate nerve terminals is blocked by ω -conotoxin GVIA and is insensitive to dihydropyridines.

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Fura-2 imaging of calcium transients in squid giant presynaptic terminal. STEPHEN J. SMITH (Yale University), LUIS R. OSSES, MILTON P. CHARLTON, AND GEORGE J. AUGUSTINE.

We employed the fluorescent Ca-indicator dye fura-2 and digital video microscopy to obtain 2-dimensional images of stimulus-induced changes in intracellular Ca in the giant presynaptic terminals of squid. These terminals were microinjected with fura-2 to a final concentration of approximately 100 μ M. Binding of Ca to fura-2 was monitored by collection of video images with fluorescence excitation at 350 nm and at 390 nm. Electrical stimuli eliciting 25-50 presynaptic action potentials were used to open presynaptic Ca channels and allow Ca to accumulate in presynaptic cytoplasm. Laterally disposed terminals were used to permit visualization of both longitudinal and lateral gradients of intracellular Ca. Abrupt longitudinal Ca gradients were observed at the boundary between presynaptic axonal and terminal regions, with Ca signals at least 10 times larger in the terminal than in the axon. This indicates that Ca channels opened by presynaptic depolarization are more concentrated in terminals than in the axon. In addition, steep lateral gradients of Ca accumulation were observed, with the larger Ca signals being observed in cytoplasm immediately adjacent to the synaptic cleft. These observations imply that the Ca channels are localized to the immediate zone of contact between pre- and post-synaptic neurons. Thus, Ca channels appear to have a restricted distribution in the presynaptic membrane, being most abundant at sites of transmitter release.

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Morphological characterization of isolated, concentrated nerve endings of the skate electric organ. H. STADLER (Max Planck Inst. F. Biophysik. Chemie, Goettingen, FRG), G. Q. FOX, G. D. PAPPAS, AND M. E. KRIEBEL.

Skate (*Raja erinacea*) electric organ isolated from the tail by blunt dissection and incubated in 1% collagenase produces homogeneous fractions of component nerve endings and electrocytes. Two hours of enzyme treatment result in the separation of the nerve plexus from the cup-shaped electrocyte. However, the electrocyte continues to elicit miniature end plate potentials (MEPPs) indicating that the presynaptic terminals remain in functional contact with the cell. An additional two hours of treatment eliminates the MEPPs and produces an electrocyte free of terminals as determined by electron microscopy. The presynaptic terminals are covered or capped by a thin Schwann cell layer and are released from the electrocyte by the collagenase as "strips" of synaptosomes. The presynaptic surface of the synaptosomes remains uncovered and represents approximately 20% of the synaptosomal plasma membrane. If cytochalasin is added to the collagenase incubation medium, MEPPs of longer duration are observed. This may indicate that cytochalasin promotes a more gradual separation of the entire efferent nerve. Attention is now focused on developing means by which to completely remove the Schwann cell cap.

Kinetics of two calcium channel types in chick sensory neurons. D. SWANDULLA AND C. M. ARMSTRONG (Univ. of Pennsylvania, Philadelphia, PA).

We studied whole cell Ca and Ba currents in chick dorsal root ganglion cells which were kept 6 to 10 h in culture. Voltage steps were imposed on the membrane in 15 μ s using an improved patch clamp circuit (Armstrong and Chow 1987, *Biophys. J.* 52: 133-136), and changes in membrane current were measured 30 μ s after the initiation of the step. Tail currents associated with Ca channel closing decayed in two clearly distinct phases which could be well fitted with two exponentials. The time constants for the tail components were ~ 160 μ s and ~ 1.5 ms at -80 mV, 20°C. The slow tail component inactivated almost completely as the test pulse duration increased up to 100 ms. It was strongly reduced when changing the holding potential from -80 to -40 mV or when adding nickel (100-200 μ M) to the external medium. This behavior indicates that the slow tail component is due to closing of the low voltage activated (l.v.a.) Ca channels (Carbone and Lux 1984, *Biophys. J.*, 46: 413-418). The fast tail component was fully activated with 10 ms test pulses to +20 mV, 20°C, and inactivated to roughly 30% during 500 ms pulses. It was hardly affected by micromolar concentrations of nickel, and only reduced in amplitude when holding at -40 mV. The time course of tail current decay did not change as the fast deactivating channels activated with short test pulses or inactivated with long ones. Neither were deactivation kinetics affected by changing the holding potential nor by varying the test pulse amplitude. Lowering the temperature from 20 to 10°C decreased the fast time constant by a factor of ~ 2.5 . In all cases the fast tail current component was very well fit by a single exponential. There was no indication for an additional exponential of significant size.

Our findings indicate that the fast tail current component in this preparation is due to closing of a single class of Ca channels which differs both in its voltage range of activation as well as its pharmacology from the well characterized l.v.a. channel type.

D.S. is supported by the Max Kade Foundation. C.M.A. is supported by NIH grant NS 12543.

VISION

Rapid desensitization terminates the response of Limulus photoreceptors to brief injections of inositol trisphosphate. RICHARD PAYNE AND ALAN FEIN (Marine Biological Laboratory).

The rhabdomeral (R)-lobe of *Limulus* ventral photoreceptors contains stores of calcium that can be released by inositol 1,4,5 triphosphate (InsP₃). The released calcium activates a conductance in the plasma membrane, depolarizing the cell. Photoreceptors were impaled in their R-lobes with pipettes containing 100 μ M InsP₃. A brief (<100 ms) pressure injection of InsP₃ delivering approx. 1 pl was followed by a second injection 1-30 s later. The first injection caused a smooth, transient depolarization of 20-45 mV. The depolarization had typically declined to 10% of its peak value within 1 s, at which time a second injection of InsP₃ was ineffective. Thus the decline of the depolarization may be due to desensitization to InsP₃, in addition to its dilution and metabolism. The amplitude of the depolarization caused by the second injection recovered fully given longer intervals between injections, 2-5 s being required for half maximal recovery. The rapid desensitization to InsP₃ could be caused by depletion of calcium stores. However, replacing extracellular calcium by EGTA had no effect on the time course of recovery from desensitization. Thus, if calcium stores are depleted following an injection, the rapid recovery of sensitivity is not due to

refilling using extracellular calcium. Alternatively, released calcium may feedback to locally desensitize the response to InsP_3 (Payne *et al.* 1986, *J. Gen. Physiol.* **127**: 107–126). Negative feedback is indicated by oscillatory bursts of depolarization following prolonged injections of InsP_3 . The interval between bursts of membrane depolarization, 2–20 s, is similar to that required for the recovery from desensitization.

Retinular cells in slices of the lateral eye of *Limulus* were also impaled with pipettes containing 100 μM InsP_3 . Pressure injections produced oscillatory bursts of depolarization that were diminished by light-adaptation or prior injection of InsP_3 . Thus InsP_3 acts similarly in lateral and ventral eyes.

Supported by grant EY03793.

Modulation of retinal sensitivity by putative efferent neurotransmitters. MELISSA R. SCHNEIDER AND ROBERT B. BARLOW JR. (Institute for Sensory Research, Syracuse University).

In many animals the brain forms a neural link with the eye to modulate retinal function. In *Limulus* the efferent input from a circadian clock in the brain terminates on retinal cells releasing one or more neurotransmitters. The neurotransmitter(s) mediate multiple changes in retinal structure and function that increase overall visual sensitivity. Three major changes in photoreceptor function include: (1) an increase in response per photon or gain; (2) an increase in photon catch; and (3) a reduction in spontaneous optic nerve activity. Are these changes produced by a single neurotransmitter or are multiple transmitters involved?

To answer this question we assessed the effects of various pharmacological agents on afferent optic nerve activity. In particular we assessed the effects of octopamine, octopamine antagonists, and forskolin on retinal sensitivity. We also assessed the action of octopamine antagonists on the effects of efferent neurotransmission.

We report that octopamine (10^{-6} – 10^{-3} M) and a potent octopamine antagonist NC-5 (10^{-5} – 10^{-3} M), when injected subcutaneously into the lateral eye *in situ*, increased gain and photon catch without reducing spontaneous optic nerve activity. The increase in gain was complete within 10 minutes of the injection whereas the increase in photon catch required at least 45 minutes to reach completion. Forskolin (10^{-5} – 10^{-4} M), an adenylate cyclase activator, also increased gain and photon catch without changing spontaneous activity. Metoclopramide (10^{-4} M), an octopamine antagonist (type 2A), completely blocked the effects of both octopamine and NC-5. Phentolamine (10^{-4} M) partially blocked the effects of octopamine and completely blocked those of NC-5. Yohimbine (10^{-3} – 10^{-4} M) did not block the effects of octopamine.

Our results indicate that the three retinal properties of gain, photon catch, and spontaneous activity can change independently of one another. Gain and photon catch can increase without a simultaneous reduction in spontaneous activity. More than one neurotransmitter may therefore mediate the circadian changes in retinal structure and function.

Supported by the Senior Fellowship Program of Hamilton College, Clinton, NY, National Science Foundation grant BNS 8320315, and National Institutes of Health grant EY-00667.

Aluminum fluoride and GTP increase inositol phosphate production in distal segments of squid photoreceptors. SUSAN F. WOOD (Marine Biological Laboratory), ETE Z. SZUTS, AND ALAN FEIN.

Light releases inositol triphosphate (IP_3) in squid photoreceptors (Szuts *et al.* 1986, *Biochem. J.* **240**: 929–932). This release may be mediated by a GTP binding (G-) protein which activates a phospholipase. To test for the role of a G-protein, stimulation of inositol phosphate production in the dark by aluminum fluoride was measured. Distal segments (labeled with ^3H -inositol) were shaken off in an artificial cytosol containing dithiothreitol, protease inhibitors, and 10 mM EGTA. This suspension was filtered, pelleted at $13,000 \times g$ for 1 min and resuspended in the above solution plus 10 mM 2,3 diphosphoglycerate (2,3 DPG), an IP_3 phosphatase inhibitor. EGTA was lowered to .1 mM for the aluminum fluoride experiments. Distal segments were incubated in the dark in 100 μM AlCl_3 and 10 mM KF at room temp. for 2–15 min. Under these conditions, the AlF_4^- form will predominate (Goldstein 1964, *Anal. Chem.* **36**: 243–244). This form activates G-proteins (Bigay *et al.* 1985, *FEBS* **191**: 181–185). After 5–15 min incubation with AlF_4^- , all inositol phosphates (IP_1 , IP_2 , and IP_3) increased. Of the lipid precursors, only phosphatidylinositol bisphosphate (PIP_2) decreased in response to AlF_4^- , suggesting a possible source of the increased inositol phosphates. The possibility that AlF_4^- could act as a phosphatase inhibitor was tested by incubating distal segments with ^3H - IP_3 in the presence or absence of AlF_4^- . No effect on the breakdown of IP_3 and IP_2 was seen even in the absence of 2,3 DPG.

Endogenous levels of GTP under these conditions are estimated to be ~ 1 μM (as measured by HPLC). This is sufficient for a 3–4 fold increase in IP_3 after a light flash. Added GTP (1 mM) caused an increase in the light response. IP_3 and IP_2 increased by $28\% + 6\%$ and $14\% + 7\%$ ($x + \text{SE}$, $n = 8$), respectively over

that seen in the absence of GTP. These results suggest that a G-protein may be involved in the light-stimulated production of IP_3 .

Near-UV light effects on the dogfish (Mustelus canis) lens. SEYMOUR ZIGMAN (University of Rochester School of Medicine, Rochester, NY 14642) AND KRIS LOWE.

Dogfish (*Mustelus canis*) were used to assess the effects of near-UV radiation on lenses with no prior exposure history. Fresh lenses were incubated in elasmobranch Ringer's solutions with 95% air:5% CO_2 for 18 h while being exposed through a pyrex beaker to near-UV radiation (365 nm max; 3 mW/cm²) from a Woods lamp. Two known near-UV sensitizers, riboflavin or tetracycline (0.5 mM), were added in some experiments; dark controls were also studied. Lenses were blotted and weighed before and after incubation. Epithelium (plus capsule) and the outer cortex were dissected and homogenized in Dounce glass homogenizers. Insoluble fractions were sedimented at 10,000 rpm; high molecular weight (HMW) colloidal fraction at 30,000 rpm; water-soluble lens crystallins remained in the supernatant (TSP). Protein contents were estimated spectrophotometrically. Polyacrylamide gel electrophoresis (PAGE) on 10% gels with 1% SDS + 50 mM DTT buffer to dissolve proteins revealed the protein subunits in each fraction. No consistent change in lens weight relative to UV-exposure with or without sensitizers was observed. UV-exposed lenses with and without sensitizers developed a diffuse subcapsular opalescence. In the cortex, UV-radiation caused a decrease in soluble, and an increase in HMW and insoluble proteins. This was exaggerated by added sensitizers. Insoluble to soluble protein ratios increased in the epithelium, especially with added sensitizer. Using PAGE, it was found that only the HMW peptide profile contained aggregated material, indicating a covalent crosslinking. We conclude that near-UV light (especially in the presence of sensitizers) leads to enhancement of crosslinked HMW proteins in the periphery. No specific crystallin was altered.

Support: Research to Prevent Blindness, Inc.; Mullie and Pledger Funds (University of Rochester); NIH (EY 00459).

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PHYSIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF THE EGG JELLY RELEASE IN *PENAEUS AZTECUS*

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ABSTRACT

Following contact with seawater, *Penaeus aztecus* ova undergo a massive release of extracortical jelly precursor material which is transformed into a layer of jelly-like material surrounding the ova. Release and dissipation of the precursors can be irreversibly inhibited by the protease inhibitors N- α -p-tosyl-L-lysine chloromethyl ketone and soybean trypsin inhibitor, implicating trypsin-like proteases in the process. Treatment with the less-specific enzyme inhibitor phenylmethyl sulfonyl fluoride also irreversibly inhibits the release of the cortical material. Jelly precursor in whole mature ovaries stain positive with PAS. Staining with alcian blue reveals acid mucopolysaccharides in the investment coat of the ova but not in the jelly precursors. Precursors isolated from whole mature ovaries are approximately 25–30% carbohydrate (anthrone sulfuric acid reaction) and 70–75% protein (Lowry's and Bradford's protein determinations). No sialic acids are detected in the isolates (thiobarbituric acid assay). Trypsin is effective in dissipating the precursor isolates. Amino acid analysis reveals high ratios of cysteic acid. Significant biochemical differences between *P. aztecus* egg jelly material and sea urchin egg jelly are discussed.

INTRODUCTION

Spawning of eggs from the ovary of the penaeid shrimp into the surrounding seawater results in a dramatic and massive release of a jelly precursor from extracellular cortical crypts (Hudinaga, 1942; Clark *et al.*, 1980, 1984). This release is initiated by contact with seawater. According to the morphological descriptions of Clark *et al.* (1980, 1984) a membrane fusion event is not involved. Initially, the jelly precursor components are stored in crypts in the surface of the mature egg separated from the environment by only a thin egg investment coat (Clark *et al.*, 1980, 1984). Transformation of the jelly precursors into the jelly coat surrounding the eggs of *P. setiferus* and *P. aztecus* has been demonstrated to be a Mg^{+2} -dependent event (Clark and Lynn, 1977). The rod-shaped jelly precursors contained in ovarian eggs of *P. setiferus* were originally described by King (1948) as peripheral bodies and recognized as jelly precursors by Hudinaga (1942) and later investigators. The biochemical composition and physiology of the release of the precursors is still poorly understood. We now report new data on the physiological parameters involved in the egg jelly release and present biochemical data on the composition of the released material.

MATERIALS AND METHODS

Animal collection

Using a standard otter trawl, brown shrimp (*P. aztecus*) were collected 80–100 miles south of Galveston, Texas. Animals were transported to the laboratory in a 150-

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Abbreviations: periodic acid-Schiff, PAS; isolation medium, IM; ethylene-diaminetetraacetic acid, EDTA; soybean trypsin inhibitor, SBTI; N- α -p-tosyl-L-lysine chloromethyl ketone, TLCK; phenylmethyl sulfonyl fluoride, PMSF.

gal tank at 13–20°C. In the laboratory, gravid female shrimp were placed in aerated, inverted 5-gal carboys, and the water temperature was slowly raised to 28°C to induce spawning. Ova obtained from these animals were used for investigations on jelly precursor release.

Jelly precursor isolation

Mature ovaries suspended in an isolation medium (IM) (500 mM NaCl, 9 mM CaCl_2 , 14 mM KCl, 15 mM MgCl_2 , and 10 mM Tris, pH 7.6) containing 30% sucrose or 35% glycerol were homogenized with a Potter-Elvehjem tissue grinder. The homogenate was centrifuged ($1000 \times g$ for 5 min). The pellet was resuspended in IM, layered over IM containing 60% sucrose or 70% glycerol, and centrifuged ($8000 \times g$ for 60 min). The resulting pellet, consisting mainly of the jelly precursor, was washed four times to remove contaminant sucrose or glycerol. These isolates were either used immediately or freeze-dried and stored at -80°C . All isolates were assayed for purity using light and electron microscopy.

Protein analysis

A Lowry's total protein reaction (Lowry *et al.*, 1951) was performed on the isolated precursor (40 mg/ml) and spectrophotometrically measured on a Coleman 124 double beam spectrophotometer. Protein was quantitated using a coomassie blue stain for total proteins (Bradford, 1976) and measured spectrophotometrically as above. Serum albumin was used as a standard for both protein measurement techniques. Amino acid analysis was performed on freeze-dried isolates. Samples were acid hydrolyzed with HCl or performic acid (Moore and Stein, 1954) and assayed on a Durum D500 amino acid analyzer.

Carbohydrate analysis

To determine the presence of carbohydrates, a molisch alpha naphthol or anthrone sulfuric acid assay (Dische, 1955) was performed on isolates (40 mg/ml). An L-cysteine sulfuric acid assay (Dische, 1955) was also performed to determine the presence of hexoses, 6-deoxyhexoses, 2-deoxypentoses, pentoses, hexuronic acids, and heptoses. Standards used for this assay were: fucose, glucose, glucuronic acid, sedoheptulose, 2-deoxy-D-ribose, and ribose (Sigma). Either the Ehrlich reaction (Werner and Odin, 1952) or the thiobarbituric acid assay (Warren, 1959) was used to test for the presence of sialic acids. N-acetyl neuraminic acid (Sigma) was used as a standard (2 $\mu\text{g}/\text{ml}$ for the thiobarbituric acid assay and 200 $\mu\text{g}/\text{ml}$ for the Ehrlich reaction).

Enzymatic digestion and inhibition

Sensitivity to enzymatic degradation was tested on fresh and freeze-dried isolates at 24–26°C. Enzymes (Sigma) used were: 0.1% trypsin (bovine pancreatic) in 0.46 M Tris, pH 8.1, containing 0.012 M CaCl_2 ; 0.1% alpha chymotrypsin (bovine pancreatic) in 8 mM Tris, pH 7.8, containing 0.1 M CaCl_2 ; 0.2% aryl sulfatase (Aerobacter) in 0.2 M sodium acetate, pH 5.0; 0.02% hyaluronidase (bovine testis) in 0.1 M monosodium phosphate, pH 5.3, containing 0.15 M NaCl; 0.1% collagenase (Clostridium) in 0.05 M Tris, pH 7.5, containing 0.35 M CaCl_2 ; 0.1% lipase (Candida cylindracea) in 1.0 M Tris, pH 8.1. All solutions were prepared according to the Worthington manual (1972). Effects of these enzymes on precursor isolates were observed with light microscopy for periods up to 5 hours. As controls, precursor isolates were held for 5 hours in the buffer systems used for each enzyme.

Spawned eggs were collected in artificial seawater (Cavanaugh, 1956) containing either 0.1% soybean trypsin inhibitor (SBTI) (Sigma), 0.1% N- α -p-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma), or 0.1% phenylmethylsulfonyl fluoride (PMSF) (Sigma). Eggs were either held in these solutions for observation or returned to normal seawater within 10 minutes after treatment in these solutions for observation. Control eggs were placed in normal seawater previously filtered through a millipore (0.2 μ m) filter or held in a solution of artificial seawater (Cavanaugh, 1956).

Microscopic techniques

Ovarian tissue was dissected from either wild mature animals or animals induced to mature by bilateral eyestalk ablation (Duronslett *et al.*, 1975). These tissues were fixed in phosphate-buffered (pH 7.8) 10% formalin or Bouin's fixative (Thompson, 1966) and embedded in paraffin. Sections (5 μ m) were stained with alcian blue 8GX at pH 2.0 (Thompson, 1966), periodic acid-Schiff reagent (PAS) (Thompson, 1966), aldehyde fuchsin (Thompson, 1966), or mucicarmine (Thompson, 1966).

Spawned eggs and isolated jelly precursor were fixed for 1–2 hours in a 0.2 M phosphate-buffered (pH 7.5) paraformaldehyde-glutaraldehyde solution (Karnovsky, 1965) for electron microscopy. Samples were post-fixed in 0.1 M phosphate-buffered (pH 7.5) osmium tetroxide (1%) for 30 minutes, rapidly dehydrated in a graded acetone series, and embedded in a low-viscosity epoxy resin (Spurr, 1969). Sections were cut with glass or diamond knives on a Porter Blum MT2-B ultramicrotome. Thin sections were stained with saturated methanolic uranyl acetate and aqueous lead citrate (Venable and Coggeshall, 1965) and examined on an Hitachi HS-8 electron microscope.

RESULTS

Mature *P. aztecus* oocytes are approximately 265 μ m in diameter and are isolecithal (Clark *et al.*, 1980). Extracellular club-shaped jelly precursors lie within membranous invaginations of the oolemma (crypts) and are separated from the environment by a thin vitelline envelope. The substructure of the jelly precursors consists of feathery elements. Contact with seawater initiates expulsion of the precursor from invaginations or crypts. As a result of the precursor expulsion, the vitelline envelope is lifted from the oolemma and is lost. Once released, the precursor elements dissipate forming a homogenous transparent jelly layer around the oocyte.

The jelly precursor elements within the crypts of an oocyte stain with PAS, but not alcian blue; however, the vitelline envelope exhibits a positive reaction with both stains. The vitelline envelope and the precursor material do not stain with either aldehyde fuchsin or mucicarmine.

Purified precursor isolates are shown in Figure 1 at the light level and in Figure 2 as observed with electron microscopy. The jelly precursor from each crypt maintains its structural integrity after isolation. Even the feathery substructural units of the precursor, originally described by Clark *et al.* (1980), are still apparent in fresh and freeze dried isolates (Fig. 3). Isolated precursor material was stable in several solvents (Table I). Sulfuric acid and sodium hydroxide completely dissipated the isolates and were compatible with the biochemical assays.

Table II shows assays for protein and carbohydrate components. The Lowry and Bradford assays were positive. From these determinations and the amino acid profiles, lyophilized precursor isolates are approximately 70–75% protein by weight. Both HCl-hydrolyzed and performic-acid-hydrolyzed isolates were analyzed for amino acid content since HCl hydrolysis partially or completely destroyed methio-

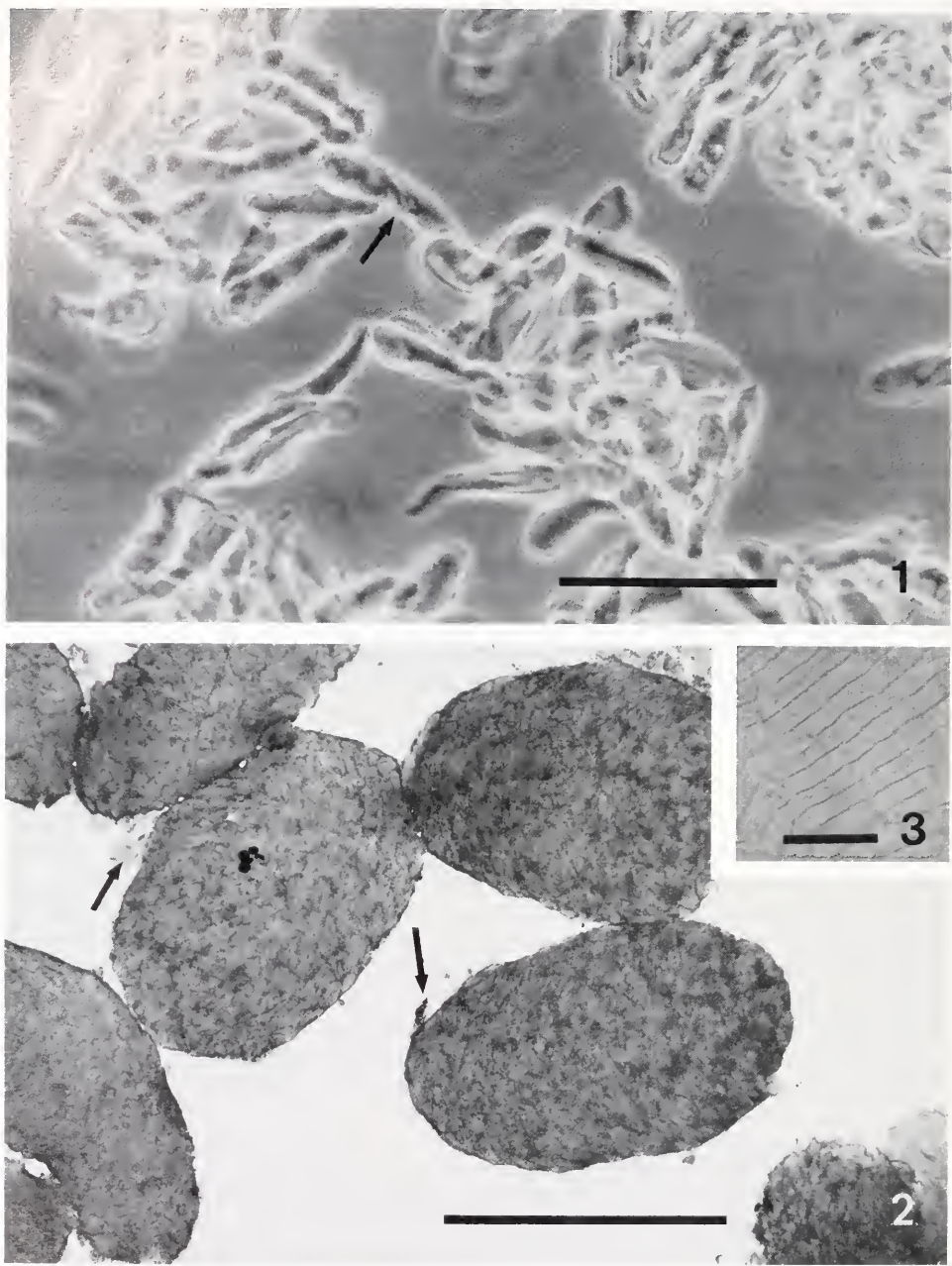


FIGURE 1. Light micrograph of a rehydrated sample of a purified jelly precursor isolate. Arrow: individual precursor element. Bar = 100 μ m.

FIGURE 2. Transmission electron micrograph of rehydrated purified precursor isolates used for the biochemical assays. Only small amounts of contaminant were apparent and appeared to be primarily yolk in nature. CR: precursor element; arrow: suspected contaminant. Bar = 10 μ m.

FIGURE 3. High magnification transmission electron micrograph of feathery substructural elements of isolated jelly precursor. Bar = 0.5 μ m.

TABLE I

Solvent extraction of jelly precursor isolates

Solvent	Precursor dissipation	Absorption peaks of solutes
8 N NaOH	Complete	260 nm, 225 nm
2 N NaOH	Complete	270 nm, 220 nm
18 N H ₂ SO ₄	Complete	465 nm, 370 nm
		320 nm, 250 nm
12 N HCl	Partial*	280 nm, 220 nm
2 N HCl	Partial*	280 nm, 220 nm
PO ₄ buffer, pH 5.8	Partial*	265 nm, 250 nm
Acetone	None	None
Chloroform	None	None
Petroleum ether	None	None
Mercaptoethanol, pH 8.8	None	None
Methanol	None	None
Triton X-100 (1%)	None	None

* Swells and becomes flocculent.

nine and cysteine. Amino acid analysis showed a predominance of aspartic acid and glycine with relatively high ratios of cysteine (Table III).

The molish alpha naphthol and the anthrone sulfuric acid assays indicated carbohydrates. Specific carbohydrate groups in the isolates were assayed using the L-cysteine sulfuric acid assay. With this assay, an initial peak in the sample was observed at 396 nm (Fig. 4), indicating a 6-deoxyhexose and possible overlapping absorption due to hexoses or pentoses. Following the addition of water and a 6-h waiting period, peaks were observed at 510 nm, 460 nm, and 410 nm. The peaks at 510 nm and 460 nm suggested heptoses. The peak at 410 nm probably resulted from a shift in the absorption of light in the range indicating a 6-deoxyhexose, since the addition of water destroys hexose absorption (Dische, 1955). Hexoses were suggested, however, by the decrease in the absorption at 396 nm after the addition of water. The sample probably did not contain pentose or 2-deoxyhexose, for no peaks comparable to the standards were observed. Sucrose contamination resulting from the isolation proce-

TABLE II

Summary of biochemical assays on jelly precursor isolates

Assay	Compounds reacting	Assay results
Lowry's	Protein	+
Bradford's	Protein	+
Molisch alpha-naphthol assay	Carbohydrates	+
Anthrone sulfuric	Hexoses, pentoses, 6-deoxyhexoses,	+
acid assay	Hexuronic acid, heptoses	+
L-cysteine sulfuric	Pentoses	—
acid assay	Hexuronic acid	—
	Hexoses	+
	Heptoses	+
	6-Deoxyhexoses	+
	2-Deoxypentoses	+
Ehrlich reaction	Neuraminic acid	—
Thiobarbituric acid assay	Neuraminic acid	—

TABLE III

Amino acid composition of isolated jelly precursor

Amino acid	Composition, moles/100 mg
Aspartic acid	126.1
Threonine	57.4
Serine	50.6
Glutamic acid	61.9
Proline	28.7
Glycine	72.4
Alanine	51.0
Valine	57.1
Methionine	13.3
Cysteine	30.8
Isoleucine	30.0
Leucine	61.2
Tyrosine	20.7
Phenylalanine	29.4
Histidine	13.9
Lysine	37.3
Arginine	18.9

ture was discounted as the source of carbohydrates because the results remained the same for preparations isolated over glycerol. A glycerol standard gave no peaks with the L-cysteine reaction. Carbohydrate content was estimated to be approximately 25–30% of the lyophilized material weight, using the anthrone sulfuric acid assay. Assays for sialic acid were negative.

Table IV summarizes the results of enzyme treatments of isolated jelly precursor elements and shows that trypsin effected complete element dispersal. Light microscopy revealed a period of swelling followed by a loss of element morphology. After a 5-h treatment with chymotrypsin, the isolates swelled but did not dissipate completely. Jelly precursor elements held in the same buffers used with the enzyme assays maintained a normal morphology and did not swell or dissipate. In addition, jelly formation was inhibited in eggs spawned into seawater containing either SBTI, TLCK, or PMSF and the effect was not reversible on return to normal seawater. If 0.1% SBTI was added to seawater that contained eggs already in the process of jelly precursor element expulsion or dissipation, jelly formation was inhibited.

DISCUSSION

The oocytes of penaeid shrimp contain a jelly-like precursor in crypts of the oolemma prior to spawning (Clark *et al.*, 1980, 1984). This material is released at spawning and forms a jelly investment around the egg (Hudinaga, 1942; Clark *et al.*, 1980, 1984). In contrast to the jelly layers that invest the ova of many animals, the penaeid coat is a primary investment produced by the oocyte (unpub. data). The penaeid material also differs in its biochemical properties from the jellies of other animal ova. Thus, while the *P. aztecus* coat may be analogous to other egg jellies, it does not appear to be homologous. While there are several biochemical differences between the penaeid shrimp jelly and the jellies in other animal ova, it must be noted that the biochemical characteristics reported in this paper deal with a precursor form of the penaeid jelly. Subtle changes in the bonding and components may occur as the heterogeneous form of the jelly is transformed into the translucent homogenous form.

Jellies of spawned eggs from sea urchins and amphibians are composed of gly-

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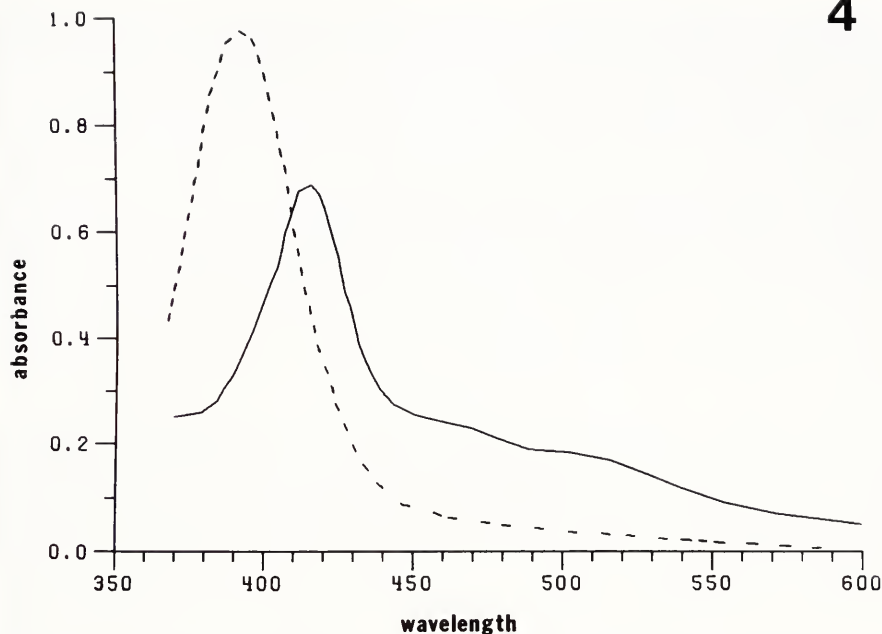


FIGURE 4. Spectrophotometric tracing of wavelength scan for the L-cysteine sulfuric acid reaction. Dotted line represents initial reaction; solid line represents reaction after a 6-hour waiting period and the addition of water. Tracing is a computer digitized reconstruction of the original scan.

cosaminoglycans, sulfate esters, and sialic acids (Vasseur, 1948; Monne and Slaughterback, 1950; Humphries, 1966; Lee, 1967; Freeman, 1968; Isaka *et al.*, 1970; Hotta *et al.*, 1970a, b, 1973, 1977; Ishihara *et al.*, 1973; Katagiri, 1973; Lorenzi and Hedrick, 1973; SeGall and Lennarz, 1979). The jelly material released from teleost and nereid eggs is also reported to contain acid mucopolysaccharides with sulfate esters and, occasionally, a neutral mucopolysaccharide (Costello, 1949; Yamamoto, 1956; Raven, 1961; Yamamoto, 1961).

Biochemical assays suggest that, unlike most other egg jellies, the penaeid jelly precursor contains a substantial amount of protein compared to carbohydrate (70–

TABLE IV

Enzymic treatments of jelly precursor isolates

Enzyme	Concentration	Effect on isolated rods
Trypsin	0.1%	++
alpha-Chymotrypsin	0.1%	+
aryl-Sulfatase	0.2%	—
Hyaluronidase	0.02%	—
Collagenase	0.1%	—
Neuraminidase	0.1%	—
Lipase	0.1%	—
alpha-Amylase	0.1%	—

++ dissipated; + swelling, no dissipation; — no effect.

75% protein, 25–30% carbohydrate). Alcian blue staining indicated carboxylated and/or sulfated carbohydrate groups in the vitelline envelope but not in the precursor material. Sialic acid was conspicuously absent from the isolates when tested by the method of Warren (1959), whereas it may compose over 25% of purified samples of sea urchin egg jelly (Hotta, 1977). In addition, enzymes capable of dispersing sea urchin egg jellies (aryl sulfatase and neuraminidase) had no detectable effect on the material released from *P. aztecus* eggs. The significance of these differences is unclear.

P. aztecus egg jelly also differs strikingly in protein content from sea urchin jelly. Sea urchin egg jelly contains approximately 20–25% protein (for review see Hunt, 1970) whereas *P. aztecus* egg jelly contains approximately 70–75% protein. Amino acid ratios, however, are similar in the two animals with the exception that the penaeid jelly material has higher cysteic acid ratios. Although sulphydryl linkages are present in penaeid jelly material, they do not appear to be primarily responsible for structural integrity, since sulphydryl reducing agents do not dissipate the jelly precursor.

Two lines of evidence indicate that trypsin-like protease enzymes are involved in the *in vivo* release and dispersion of the jelly precursor. Isolates were effectively dissipated by trypsin and to a much lesser degree by chymotrypsin. Secondly the specific serine-protease inhibitor SBTI inhibited this release and dispersion *in vivo*. Although TLCK and PMSF may also inhibit SH proteases in addition to serine proteases (Whitaker and Perez-Villaseñor, 1968), a serine protease is preferentially supported since SBTI is not reported to inhibit SH proteases and it is capable of completely inhibiting the jelly release. Regardless, a trypsin-like enzyme is suggested, but isolation and *in vitro* characterization of the enzyme will be essential in identifying the specific class of proteases represented by these enzymes.

Proteases are additionally implicated by the ability of the inhibitors to stop jelly formation at two stages: when the precursors are released from their crypts, and later, when the material is transformed into a translucent jelly layer. It is particularly interesting that these two stages of jelly release and transformation are also completely inhibited by a deficiency of Mg^{+2} in the seawater (Clark and Lynn, 1977) suggesting that the proteases involved are also Mg^{+2} -dependent. A similar protease-dependent jelly precursor release and transition of the jelly precursor into a homogenous jelly layer has also been observed in the eggs of *Sicyonia ingentis* (unpub. data). The initial phase of release which appears to be less Mg^{+2} -sensitive in the *S. ingentis* egg, seems to be mediated by failure of the vitelline envelope to break down, preventing jelly expulsion. A similar phenomenon may be responsible for preventing jelly release in the *P. aztecus* egg. The location of the proposed protease involved in the penaeid egg release is unknown. Therefore, specific activities of the enzymes involved must be localized and described to understand the sequence of events of the egg jelly release.

Despite the differences in chemical composition between egg jellies reported in other species and the jelly formed by *P. aztecus*, it is likely that the different types of jelly layers in question may have very similar functions. For example, the jelly formation in *P. aztecus* may protect the early zygote from the environment. Although morphologically the precursors are rapidly dissipated, their chemical constituents may remain around the ova for a long time. In this form, these chemical constituents could act as an antibacterial agent or as a repellent to other microorganisms. Preliminary studies reveal zones of inhibition around jelly precursor isolates placed on freshly streaked plates of shrimp exoskeletal bacteria (unpub. data). This exciting possibility should be further pursued *in vivo*.

An additional or alternative role for the formation of the jelly layer in *P. aztecus* may involve the acrosome reaction of the sperm. Acrosomal inducing abilities of egg investment coats have been demonstrated in sea urchins (SeGall and Lennarz, 1979).

and sturgeon (Cherr and Clark, 1985). Indeed, the investment coats of many species may contain components which trigger the acrosome reaction in sperm (Lopo, 1983, for review). Although the role of the *P. aztecus* jelly as an acrosomal reaction inducer has not been tested, a component of egg jelly from a related penaeid shrimp, *S. ingentis* has recently been demonstrated to induce the acrosome reaction (Clark *et al.*, 1984).

This study shows that the jelly formation in *P. aztecus* differs markedly from the cortical reactions of other animals, such as sea urchins. Instead, this reaction appears to be a delayed jelly-coat formation. It remains to be seen whether the physiological and biochemical differences of the shrimp jelly coat indicate, reflect, or match functional similarities of jelly investments of other animals.

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QUANTITATIVE GENETICS OF JUVENILE GROWTH AND SHAPE IN THE MUD CRAB *EURYPANOPEUS DEPRESSUS*¹

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ABSTRACT

Rates of growth and development were measured for the first six molts following the crab I stage in the mud crab *Eurypanopeus depressus*. The genetic contribution to variation in growth rate, development rate, and shape was determined for each molt interval. Genetic variation in growth rate, measured as increases in both width and length, was evident at most molt intervals. There were also significant genetic effects upon the intermolt interval. Growth rates for each molt interval, calculated on a daily basis to remove the interaction between growth rate and development rate also showed genetic variation. There was no evidence that genetic variation in these parameters changed during early juvenile development; there were substantial levels of genetic variation in growth rate at most ontological stages. Despite high levels of genetic variation for growth rate in dimensions of the carapace, there was no evidence of genetic variation in shape. This analysis does not provide a quantitative estimate of the levels of genetic variance for these traits but does indicate that the magnitude of this source of variance must be very significant.

INTRODUCTION

Variance in many traits may have a large genetic component. High heritabilities have been demonstrated for many traits, including morphology (van Noordwijk *et al.*, 1980; Boag, 1983), behavior (Arnold, 1981a, b; Via, 1984a, b), physiology (Curtis-singer and Laurie-Ahlberg, 1981), and other traits that are ecologically important and have a strong influence upon fitness. Current studies focus on understanding the role of development in genetic variation for these traits. It is important to determine whether quantitative genetic variation for a trait is stable throughout the development of an organism. If the heritability of a trait changes during ontogeny, then natural selection can only influence the trait during intervals of high heritability. Conversely, if natural selection only occurs during certain periods of development, then the trait will be more free to vary during other portions of ontogeny.

The mud crab *Eurypanopeus depressus* (Smith) has several features which will make it suitable for a quantitative analysis of growth and development. Like other arthropods, changes in size and shape in *Eurypanopeus* is restricted to a short interval following a molt while the new exoskeleton is still flexible. In addition, molting of the exoskeleton uniquely defines developmental events. In many nonarthropod species the accurate assessment of developmental progress or developmental staging is either difficult or is a largely arbitrary process. Accurate developmental assessment is essen-

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tial to an understanding of changes in heritability during ontogeny. Finally, in *Eurypanopeus*, early juvenile development includes a rapid change in shape. During the first three juvenile molts, these crabs transform from a megalops with a square carapace to the trapezoidal shape typical of the adult stage in this species. These characteristics allow us to address several fundamental questions on the quantitative genetics of juvenile growth and form. We will determine the relative importance of genetic causes to the variance in early juvenile growth and shape in *Eurypanopeus*. We will also determine whether genetic variation in these traits varies during development and whether the genetic component of variance changes during the transformation in shape that occurs early in juvenile development.

MATERIALS AND METHODS

Eurypanopeus depressus is the most common mud crab inhabiting oyster reefs along the east coast of the United States (Williams, 1984) and is abundant in intertidal oyster reefs in South Carolina. Ovigerous females are found from April to November in the North Inlet estuary (Georgetown, South Carolina). Costlow and Bookhout (1961) described the larval development of this species reared under laboratory conditions. They reported four zoeal stages and a megalops.

Gravid *Eurypanopeus depressus* females were collected at the Baruch Institute Field Laboratory near Georgetown, South Carolina, during the summer of 1978. All females used in this study were collected at one time. Each female was maintained in the laboratory at 25°C and a 14:10 h light:dark cycle in an individual bowl of 30‰ seawater until an egg mass was expelled. Egg clutches from six females were hatched and individually reared in beakers through the zoeal stages. Zoeae were maintained under the same environmental conditions as their mothers and were fed freshly hatched *Artemia ad libitum*.

Upon metamorphosis to the megalops stage, juveniles crabs were transferred to individual cells in a compartmented box where they were maintained with seawater (30‰ and 25°C). To reduce the effects of a shared environment, the compartmentalized boxes were returned to the incubator in a random placement following a daily change in seawater. Juvenile crabs were fed excess amounts of freshly hatched *Artemia* daily. Individual crabs were checked daily for a molted exoskeleton. Upon molting the exoskeleton was removed and the crab was measured for both carapace length and width to the nearest 0.01 mm using an ocular micrometer on a dissecting microscope. Carapace length was measured as the distance between the anterior and posterior margins of the shell. Width was measured as the maximal distance across the lateral margins of the shell. The number of days elapsed since metamorphosis to megalops was recorded at each molting. During the experiment individual animals died, so sample sizes vary throughout the analysis. Measurements continued for eight successive molts but numbers declined significantly after the sixth molt. Therefore, the analysis reported here is restricted to the first six molts.

Genetic and statistical analysis

Data were collected such that members of the same family could be distinguished throughout the analysis. Quantitative genetics uses the similarity among relatives to determine the proportion of the phenotypic variance in a trait that can be explained by genetic variance (Falconer, 1981). Therefore, if the mean value for a trait, such as body size, varies significantly among families this implies that close relatives may

appear similar because of the genes they share in common. It is also possible that relatives are similar in appearance for non-genetic reasons. These possibilities are discussed below. In this analysis we know whether two individuals share the same mother; we do not know whether they share the same father. Therefore it is unknown whether offspring derived from the same clutch are full- or half-siblings. Without knowing the exact genetic relationship among siblings it is impossible to estimate heritability (genetic variance/total phenotypic variance) accurately. However, we can estimate whether there is a significant genetic effect upon the phenotypic variance observed in these samples of juvenile crabs. This analysis is similar to that used in other studies of quantitative genetics using wild-caught pregnant females (Arnold, 1981a, b).

Relative growth rates were determined by regressing the change in size between successive molts against initial size for each individual within a family. Variation among families in relative growth was then analyzed using analysis of covariance. Relative growth rates are reported as mm growth/mm initial size. Relative growth rates were determined for both length and width and were calculated on a per molt and per day basis (see below). Development rate was calculated as the number of days required to proceed from one molt to the next. Variation among families in development rate was analyzed using ANOVA. *Eurypanopeus depressus* varies dramatically in shape during juvenile development. Shape can be expressed as the ratio of width to length. However, ratios have unusual sampling distributions and ANOVA is not an appropriate methodology for their analysis (Atchley *et al.*, 1976). Therefore, carapace length was regressed against width for each family and analysis of covariance used to determine whether there was significant variation among families in either slopes or adjusted means of the regressions.

Relative growth rates are expressed in two ways. First, the relative growth between two successive molts was determined by regressing the change in size against initial size for each family. Variation among families was then analyzed using ANCOVA. The analysis of covariance reports the average change in size for each family adjusted to the average initial size for all families. Relative growth rates were then calculated by dividing each adjusted mean by the average initial size. Therefore, relative growth rates are reported as mm growth/mm initial size/molts (mm/mm/molts). This method of expressing relative growth indicates the change in size between molts but does not account for the amount of time required to proceed from one molt to the next. Therefore growth rates were also calculated by determining the change in size between successive molts for each individual and dividing by the intermolt interval for that individual. This quantity was then regressed against initial size to determine relative growth rates and the data analyzed by ANCOVA.

The family means for all growth and development rate measurements are presented in graphical form. It was not possible to simultaneously present the standard errors of the means for these analyses without obscuring the graphical presentations. Therefore the following convention was adopted to provide a representation of the variance about each family mean. The error mean square is reported for each analysis of variance in tabular form. Combined with the sample sizes for each family these data may be used to calculate either standard errors or confidence limits for the family means (Sokal and Rohlf, 1981). The six families used in this study varied in size. In addition, some individuals died and occasionally individual measurements were lost. Therefore sample sizes within a family vary slightly from one experiment to the next. Average sample size and the range in sample size for each family are: family 1, 10.2

TABLE I

F-tests for variation among families in growth and development rates. Error mean squares are also reported for each test

	Molt interval				
	1-2	2-3	3-4	4-5	5-6
GROWTH RATE					
Length/Molt					
F-value	3.61 (102)**	3.29 (110)**	1.98 (112)	2.14 (107)	1.83 (84)
error MS	0.0098	0.0085	0.0170	0.0079	0.0058
Width/Molt					
F-value	3.21 (112)**	1.65 (117)	6.20 (115)***	0.23 (112)	1.65 (89)
error MS	0.0137	0.0100	0.0075	0.0041	0.0044
Length/Day					
F-value	4.00 (94)***	0.54 (112)	0.92 (112)	2.64 (107)*	4.09 (78)**
error MS	0.00105	0.00105	0.00047	0.00023	0.00005
Width/Day					
F-value	4.75 (102)***	0.74 (117)	4.56 (115)***	1.62 (112)	3.33 (88)*
error MS	0.00105	0.00104	0.00023	0.00018	0.00009
DEVELOPMENT RATE					
F-value	4.10 (110)***	1.25 (120)	3.29 (119)**	2.70 (115)*	6.04 (94)***
error MS	5.99	2.02	4.29	24.95	17.53

The denominator degrees of freedom are reported in parenthesis. The numerator degrees of freedom are equal to 5 in all cases. The significance of the *F*-test is indicated by an asterisk (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). *F*-values without an asterisk are not significant at the 5% level.

(7-12); family 2, 13.5 (11-15); family 3, 17.1 (10-20); family 4, 21.7 (18-24); family 5, 38.6 (31-42); and family 6, 12.1 (11-13).

RESULTS

Relative growth per molt

Relative increases in length varied with development and among families. Between the first and second molt the average relative growth rate was 0.25 mm/mm/molt and there was significant variation among families in their average growth rate ($P < 0.01$, Table I). Families 1, 3, 5, and 6 exhibited high growth rates of approximately 0.25 mm/mm/molt while families 2 and 4 exhibited much lower growth rates of approximately 0.17 mm/mm/molt (Fig. 1). The error mean square for relative growth rates over this first interval and all subsequent analyses are presented in Table I.

Between the second and third molt families 1, 3, 5, and 6 continued to grow at significantly higher rates than did families 2 and 4 ($P < 0.01$, Table I). The highest relative growth rates were observed between the third and fourth molt, averaging 0.36 mm/mm/molt (Fig. 1). However the variation among families was not significant during this interval. Average growth rates dropped to 0.16 mm/mm/molt in the next two molt intervals (molt 4-5 and 5-6), and there was no significant variation among families (Fig. 1; Table I). In summary, there was significant variation among families in relative growth rate for length in the first two molt intervals. There was no signifi-

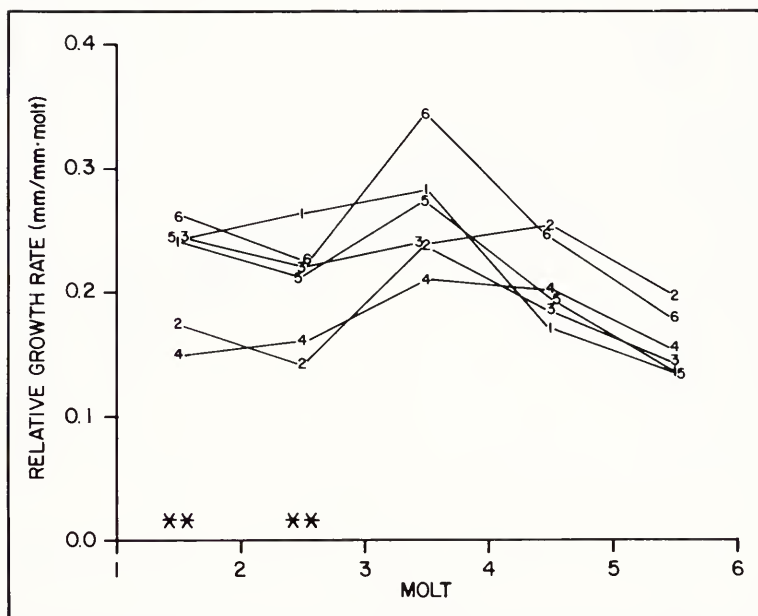


FIGURE 1. Relative growth rates in length for *Eurypanopeus depressus* for each intermolt interval. Growth rates are reported in mm/mm/molt. Each symbol indicates the mean growth rate for each of the six families used in the analysis. Significant variation among families is indicated along the abscissa with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

cant variation among families in relative growth rate as development progressed beyond the third molt.

Similar to growth in length, relative changes in width depended on both development and family. Relative growth rates were initially high, averaging 0.30 mm/mm/molt between molts 1 and 2. Between molts 5 and 6 these rates declined to an average value of 0.17 mm/mm/molt (Fig. 2). There was significant variation among families in relative growth rate between molts 1 and 2 ($P < 0.01$, Table I) and molts 3 and 4 ($P < 0.001$, Table I). Family 6 was the fastest growing group in virtually all molt intervals. Families 2 and 4 were initially the slowest growing families, but by the final molt interval they were among the most rapidly growing families. This change in relative growth rates was similar to that observed for growth in length. Variation in relative growth in width was not significant among families in any other molt interval (Fig. 2; Table I).

Relative daily growth

Relative growth rates were also calculated by dividing the change in size between molts by the interval between molts. This method of expressing relative growth provides a daily estimate of growth but also includes two potentially genetically variable parameters; growth and development time. An alternate way of viewing this expression of growth is that it removes a potential artifact in the previous expression by controlling for variation in intermolt intervals. In many species growth between molts

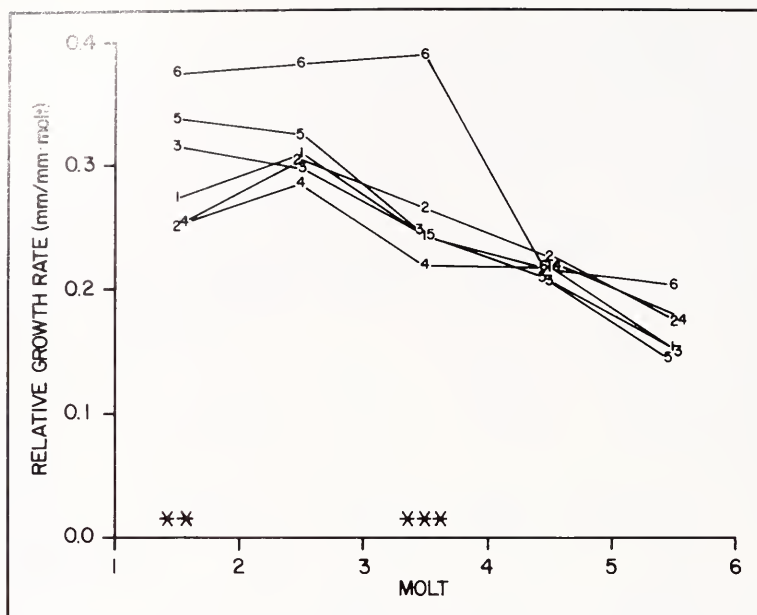


FIGURE 2. Relative growth rates in width for *Eurypanopeus depressus* for each intermolt interval. Growth rates are reported in mm/mm/molt. Each symbol indicates the mean growth rate for each of the six families used in the analysis. Significant variation among families is indicated along the abscissa with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

is a function of the length of time between molts. Therefore the previous analysis may confuse variation in the interval between molts with variation in growth rate. This artifact is minimized by expressing relative growth on a daily rather than per molt basis.

Relative daily growth in length varied during development and was strongly dependent upon family. Relative increase in length was initially high, averaging 0.049 mm/mm/day, but declined steadily with each molt (Fig. 3). The average daily growth rate was 0.015 mm/mm/day between the fifth and sixth molts. Daily growth rates in length also exhibited a significant family effect. Between molts 1 and 2, families 1, 3, 5, and 6 grew at nearly double the rates of families 2 and 4 ($P < 0.001$; Table I; Fig. 3). In the interval between molts 2 and 3 and between molts 3 and 4 there were no significant differences among families in daily growth rates (Table I). The variance in daily growth rate among families was again significant ($P < 0.05$; Table I) between molts 4 and 5. There was also significant variation among families between molts 5 and 6 ($P < 0.01$, Table I). There was a clear change in the rank order of relative growth rates among the families with progressing development. Families 2 and 4 were initially the slowest growing groups, but by the final molt interval they were the fastest growing families (Fig. 3).

Relative daily growth rates for width decreased steadily with each successive molt. Initially relative growth rates in width were 0.067 mm/mm/day and declined to 0.016 mm/mm/day by the final molt interval (Fig. 4). As with previous measures of growth, relative daily growth in width also depended upon family. Between molts 1 and 2, the variation among families was highly significant ($P < 0.001$, Table I). Between

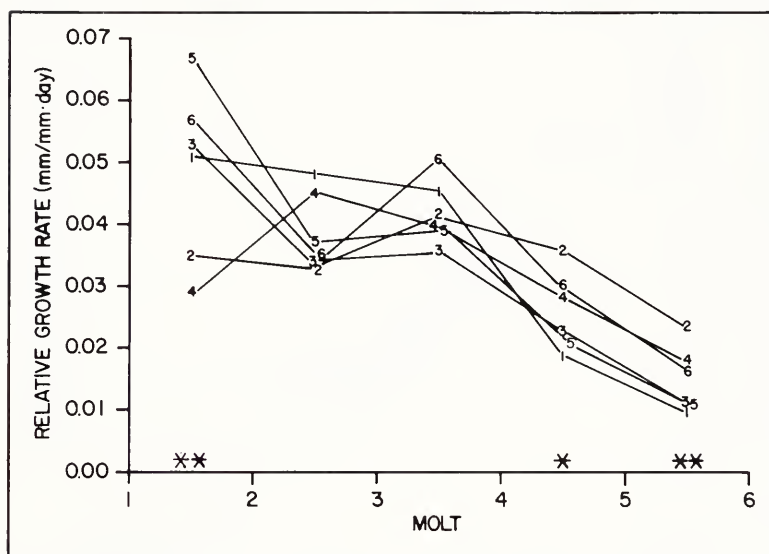


FIGURE 3. Relative daily growth rates in length for *Eurypanopeus depressus* for each intermolt interval. Growth rates are reported in mm/mm/day. Each symbol indicates the mean growth rate for each of the six families used in the analysis. Significant variation among families is indicated along the abscissa with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

molts 2 and 3 there was no significant variation among families in relative daily growth. Between molts 3 and 4 there was again significant variation among families ($P < 0.001$, Table I). Between molts 4 and 5 there was no significant variance among families while in the final molt interval the variation among family means was significant ($P < 0.05$, Table I). As with previous analyses, families 2 and 4 initially exhibited low relative growth rates and ultimately became the fastest growing families by the final molt (Fig. 4).

Development rate

The intermolt interval increased with development. The interval between the first and second molt averaged 5.2 days while it required about 12.8 days to proceed from molt 5 to 6 (Fig. 5). The variation among families changed substantially over the course of development. Early in development, between molts 1 and 2, variation among families was highly significant ($P < 0.001$, Table I). This variation was due primarily to the relatively long intermolt period of families 3 and 4 (Fig. 5). The time required to proceed from molt 2 to 3 was virtually identical in all cases; the variation among families was not significant. After the third molt, families 2 and 4 exhibited a large decrease in the intermolt interval relative to the other families. By the final molt interval these two families had an intermolt period 35% lower than the other four families (Fig. 5). The variation among families in intermolt time was also significant for the intervals between molts 3 and 4, molts 4 and 5, and molts 5 and 6 (Table I).

Shape

Carapace width initially increased at much higher rates than did length. Ultimately the rates of increase in width and length converged by the final molt interval.

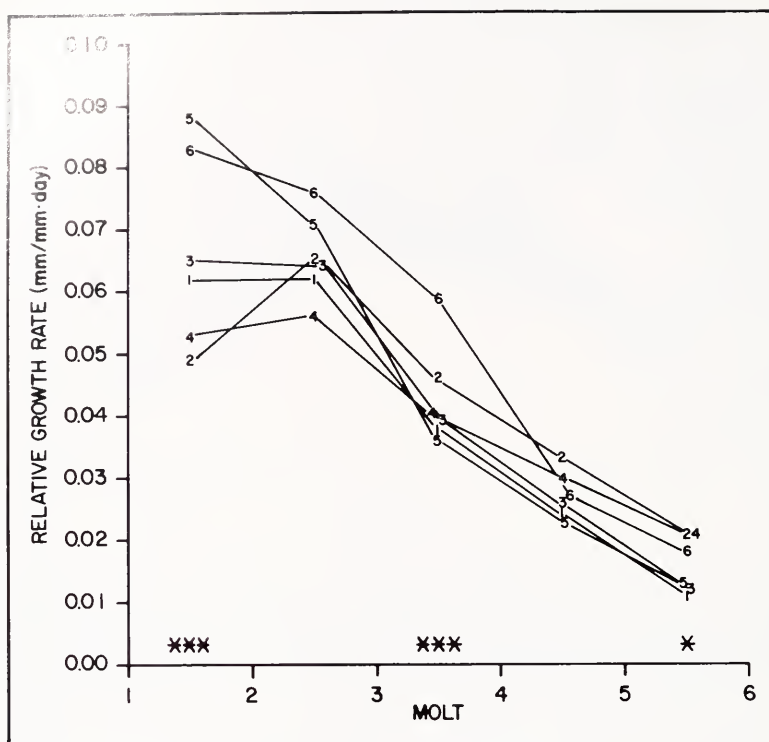


FIGURE 4. Relative daily growth rates in width for *Eurypanopeus depressus* for each intermolt interval. Growth rates are reported in mm/mm/day. Each symbol indicates the mean growth rate for each of the six families used in the analysis. Significant variation among families is indicated along the abscissa with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

This resulted in a major change in shape over the first few juvenile molts. After metamorphosis to the first crab stage the carapace of *Eurypanopeus depressus* is approximately square, with an average width to length ratio of 0.98 (Fig. 6). During the second molt the ratio of width to length increased to 1.08. The ratio increased to 1.2 during the third molt. During molts 4, 5, and 6 there was a gradual but continual increase in the width to length ratio up to an average of 1.24 (Fig. 6). By the sixth molt the crabs had adopted the trapezoidal shape characteristic of adult *Eurypanopeus*.

The analysis of shape was designed to test two questions: do families vary in shape and where in ontogeny is this source of variance of greatest importance? Carapace width was regressed against length for each family, and ANCOVA was used to determine if there was significant variation in shape among families at each molt. Significant variation in adjusted means indicates that some families are consistently broader or narrower than others. Significant variation among slopes of these regressions indicates that the manner in which width scales onto length depends upon family. Therefore, either variation in adjusted means or slopes would be indicative of shape variation among families.

Variation in adjusted means was not significant for any of the six molts (Fig. 6; Table II). With the exception of the sixth molt there were also no significant differ-

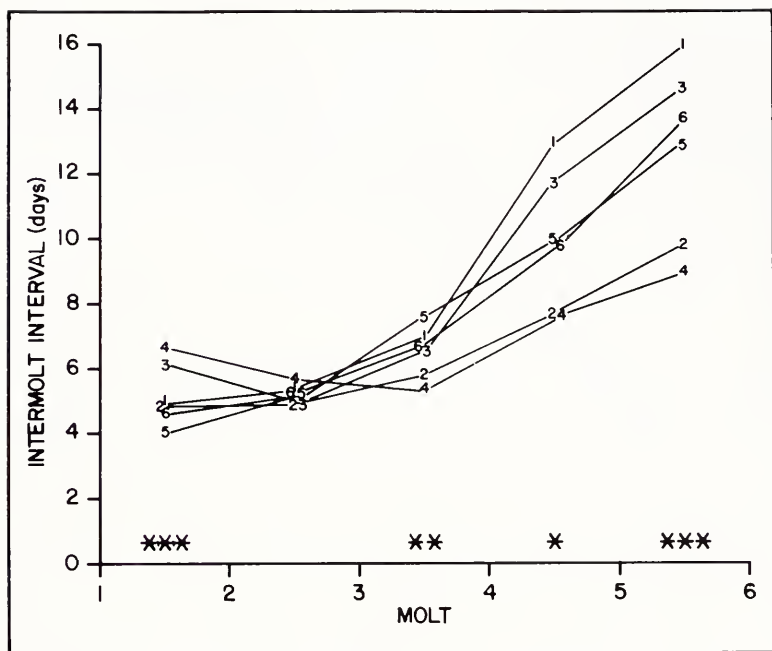


FIGURE 5. Mean development rates for six families of *Eurypanopeus depressus* for each intermolt interval. Development rates are reported as the days required to proceed from one molt to the next. Significant variation among families is indicated along the abscissa with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

ences among families in the slope of the width on length regressions (Table II). In the sixth molt there was significant variation among families in the slopes of these regressions ($0.01 < P < 0.05$). However this one case of significance may be due to random chance. There were 12 tests of significance performed in this analysis, of which one was significant at the 0.05 level. Rejections at this significance level should occur by chance 5% of the time (0.6 times in 12 tests). It should also be noted from Figures 1 and 2 that there was no significant variation among families in increases in either width or length which also suggests that the significance of variation in shape among families during this interval was spurious. In summary, there is no convincing evidence of significant variation in shape among the six families used in this analysis.

DISCUSSION

There was significant variation among families in relative growth rates. This was true for rates of increase in both width and length and for growth rates calculated on both a per molt and daily basis. Variation in growth rate was observed throughout juvenile development; every molt interval included significant family variation in at least one of the measures of relative growth rate. Natural selection must operate on traits that retain significant genetic variation and there has been considerable interest in the manner in which heritable variation may be distributed through development. For *Eurypanopeus depressus*, genetic variation for growth rate persists for all juvenile

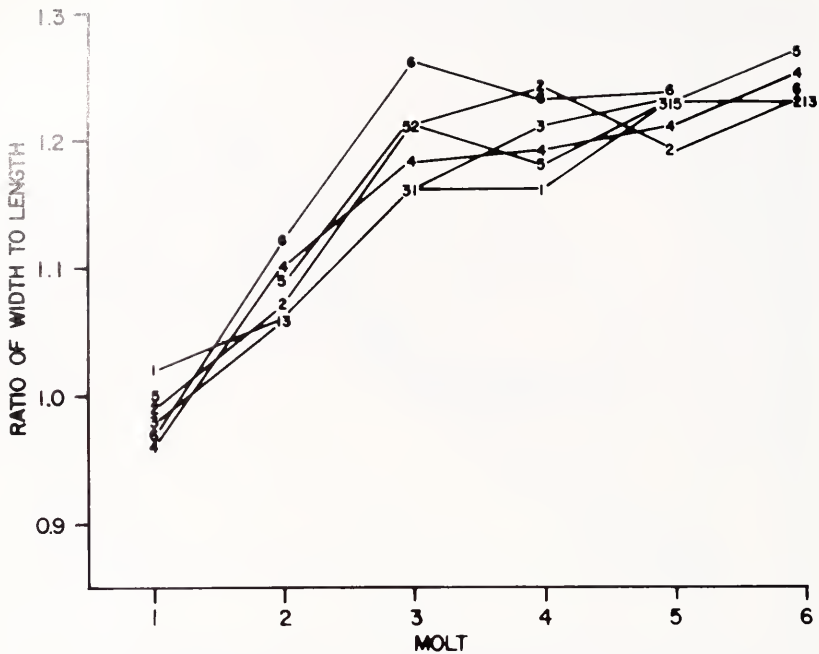


FIGURE 6. Width to length ratios for six families of *Eurypanopeus depressus* after the first six juvenile molts. Width to length ratios are mean values for each family.

developmental stages. Therefore, natural selection could potentially affect growth rate in this species at any point during its juvenile development.

The development rate also varied among families. This variation must explain in part the growth rate variation observed among families. In general, high growth rates in any given interval were inversely correlated with the duration of an intermolt interval. Rapid growth and rapid development appear to co-occur during development. However, this correlation has little bearing on the variation in growth observed

TABLE II

Results of ANCOVA of carapace width regressed against length for each family at each molt

Molt	F (adjusted means)	F (slopes)
1	1.57 (121)	1.85 (116)
2	1.32 (119)	0.76 (114)
3	1.40 (118)	1.80 (113)
4	1.26 (118)	0.53 (113)
5	0.85 (114)	1.05 (109)
6	0.89 (90)	2.59* (85)

F values for tests of homogeneity of adjusted means and slopes are given. The numerator degree of freedom was 5 in every test. The denominator degrees of freedom are given in parenthesis.

* $P < 0.05$.

among families. We correlated the growth and development rates of each family within a molt interval; there were no significant correlations between growth and development rates within any molt interval. This lack of correlation is exemplified by the interval between the second and third molt in which intermolt intervals were identical for all six families yet there was considerable variation in growth. In addition, one would expect that expressing growth rate on a daily basis would reduce any role of development rate in indirectly explaining among family variation in growth. This was clearly not the case—the expression of growth on a daily basis appeared to increase the differences among families. Therefore, it appears that families of *Eurypanopeus depressus* differ in both growth and development rates and that these interact in a complex manner.

While it is apparent that there is substantial variation among families in rates of growth and development, the origin of this variation is not obvious. Significant covariation among family members may be due to genetic causes or to a shared environment (Falconer, 1981). The most obvious source of a common environment that may lead to covariation among siblings is a maternal effect. However it is also possible that a shared environment during the larval culture phase could lead to significant elevation in the similarity among relatives. With the experimental design used here it is not possible to unequivocally reject the hypothesis of maternal effects leading to the observed variation among families. However, the pattern of growth rate variation among families indicates that maternal effects are an unlikely explanation for these results. One consistent trend in the growth data was that families 2 and 4 initially exhibited low growth rates which increased during development until in most cases they became the most rapidly growing families. The other four families were typically fast growing during the initial molts and then declined in their relative growth rates. Maternal effects might have a reasonably consistent impact on the growth of each family. For example, if a female produces high quality eggs, perhaps by the inclusion of atypically high yolk levels, the offspring born to that female should consistently grow faster than offspring produced from inferior eggs. What was observed here was a relative growth advantage of some families that changes during ontogeny. This switching of growth advantage is difficult to explain by maternal or other common environmental effects. Therefore we conclude that a significant proportion of the variation in growth and development observed among families is of genetic origin.

In a conventional quantitative genetic design, controlled matings are produced in which paternal effects can be quantified and the degree of genetic relation among offspring known. The study of genetic variation in natural populations has certain liabilities that are exemplified in this study. The first has been discussed. By using wild-caught ovigerous females the among family variation includes both genetic and maternal effects. This design is similar to that of Arnold (1981a, b) who used pregnant female garter snakes to generate families of full-siblings.

A second problem with using wild-caught pregnant females is that we are uncertain as to the genetic relationship among siblings. Individuals born to a given female may be either full- or half-siblings depending upon the mating system of the population under investigation. Many species of brachyuran crabs store sperm from multiple matings (Sastri, 1983). Therefore the offspring of a single *Eurypanopeus* female are likely to be a combination of full and half-siblings. Without knowing the exact genetic relationship among offspring, it is impossible to partition the variance in a trait into genetic and environmental sources or to subpartition the genetic variance into additive and nonadditive components. While we cannot accurately estimate the magnitude of the genetic contribution to the variation in growth and development

rates, it is clear that a substantial fraction of this variation must be genetic in origin. Family effects were very striking; in many incidences family explained greater than 20% of the total variation observed in relative growth rates. Therefore genetic variation for growth in *Eurypanopeus depressus* is prevalent but a more controlled breeding design would be required to produce an accurate estimate of its magnitude.

Genetic variation in growth has been observed in other marine invertebrates. Single or multilocus genetical analysis of enzyme-coding genes has often revealed a significant genetic component to growth (Zouros *et al.*, 1980; Garton, 1984; Koehn and Gaffney, 1984). Quantitative genetic analysis also revealed that a substantial fraction of the observed variation in growth in marine bivalves is of genetic origin (Lannan, 1972; Newkirk *et al.*, 1977, 1981). This study likewise found a large proportion of the variation in growth is due to genetic variation.

There was no evidence that shape of the carapace in *Eurypanopeus depressus* was genetically variable. This was surprising in light of the highly significant variation among families in increase in both width and length which are the two parameters used to determine carapace shape. There is increasing interest in the analysis of shape and in the role ontogeny plays in altering genetic variances in morphology (Atchley and Rutledge, 1980; Leamy and Cheverund, 1984). Recently, Riska *et al.* (1984) demonstrated that morphological traits may have high levels of quantitative genetic variation associated with them early in juvenile development, but following the attainment of adult stature this genetic variation is sharply reduced. This study on mice illustrates the commanding role ontogeny may have in the expression of genetic variation. In the present study, we expected to observe significant family effects on shape, particularly between the second and third molt when shape changed rapidly. This was not the case. There was only one incidence of significant variation among families in shape and this case was probably not meaningful. Therefore we conclude that in *Eurypanopeus depressus* growth and development rates are genetically variable while shape is a highly conserved trait, exhibiting virtually no genetic variation during juvenile development.

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THE FATE OF *BOTRYLLUS* (ASCIDIACEA) LARVAE COSETTLED WITH PARENTAL COLONIES: BENEFICIAL OR DELETERIOUS CONSEQUENCES?

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ABSTRACT

The consequences of settlement of *Botryllus* larvae close to or on parental colonies were followed in two sets of experiments. In the first, 28 experimental progeny settled adjacent to 6 parents; 207 other sibling progeny served as controls. Four different types of interactions between parent colony and offspring were observed: fusion and resorption of the offspring, fusion and separation, tunic-to-tunic contact and separation, tunic-to-tunic contact, and the death of offspring. Offspring interacting with parents had significantly higher mortality than control offspring. Resorption was the fastest process (one week on average); the two "separation" processes lasted approximately two months. Twenty of the 21 progeny that died after interacting with parents did not grow at all (even after 75 days). All 7 offspring that separated from their parents grew. In two cases of fusion between offspring and adults, large eggs were found within the progeny zooids. Presumably the eggs translocated from the maternal colony through the connecting blood vessels. Only five progeny survived in this set of experiments, a phenomenon which coincided with the degeneration or the mortality of the parent. In the second set of experiments all 93 progeny which had settled on old, dead tunics of 5 parental colonies died within 8 weeks.

These results indicate that cosettlement of offspring proximal to their parental colony is usually deleterious in the long term to the progeny, both when they fuse with or when they merely contact the parent. This phenomenon was also recorded in field observations. We suggest that the phenomenon of gregarious settlement of *Botryllus* larvae near their parents, although characterized by the loss of many progeny, is nonetheless advantageous in response to biotic interactions such as interspecific competition. In this view resorption may have evolved as a secondary process, as a result of the nature of self/nonself recognition in *Botryllus*.

INTRODUCTION

Colonies of the tunicate *Botryllus* originate from a sexually produced planktonic larva. The larva attaches to a substrate and there metamorphoses into a "founder" oozoid. Colonies arise by asexual multiplication (budding) from this "founder" oozoid. The result is a colony of morphologically and genetically identical zooids. The star-shaped groups of zooids in the colony all share a common blood-vascular system embedded in a continuous gelatinous matrix, the tunic. Studies of colony specificity show self/nonself discrimination in the genus *Botryllus* (reviewed by Taneda *et al.*, 1985). When the growing parts of two adjacent colonies of the same species come

into direct contact, they either reject one another or fuse. This histocompatibility (or fusibility) discrimination is controlled by a single gene locus with multiple codominantly expressed alleles (Oka and Watanabe, 1960; Sabbadin, 1962; Scofield *et al.*, 1982; Scofield and Nagashima, 1983). Consequently, two colonies sharing one or both alleles at the fusibility locus can fuse through their vascular blood systems. Colonies that do not share either allele at the fusibility determining locus reject each other.

Grosberg and Quinn (1986) suggested that larvae of *B. schlosseri* from Woods Hole, MA, distinguish kin on the basis of shared fusibility locus alleles, a mechanism which promotes cosettlement of histocompatible colonies. A gregarious settlement of *Botryllus* sibling larvae was also recorded from Monterey, CA (Scofield *et al.*, 1982), and in populations from the Mediterranean Sea (Sabbadin, 1978). Sibling cosettlement in *Botryllus* may be intensified rapidly by the mechanism of larval metamorphosis after larval release into the water column (Grave and Woodbridge, 1924; Sabbadin, 1978; Grosberg and Quinn, 1986). This promotes cosettlement of larvae in proximity to their parental colonies (Sabbadin, 1978; Grosberg and Quinn, 1986; our unpubl. data). Offspring share at least one allele with their parental colony at the fusibility-histocompatibility locus. Therefore they have the capacity to fuse with the parent colony.

Grosberg and Quinn (1986) proposed that colony fusion among kin may be beneficial to both members of the chimera in several ways. For example, colony fusion immediately increases the chimeric-colony size. Since survivorship and onset of reproduction are known to be size dependent, fusion might reduce the likelihood of mortality and lower the age of first reproduction. Here we describe a laboratory study of the consequences of offspring cosettlement near their natal colony. Survivorship and growth rates of the larvae were followed and compared to other, control offspring. In another set of experiments we followed the survivorship of progeny settled on their maternal-colony tunic.

MATERIALS AND METHODS

Eleven large sexually matured colonies of Monterey *Botryllus schlosseri* were used in two sets of experiments. The sexual maturity of each colony was determined by embryos developing inside the zooids. All the large colonies were born and raised in the laboratory in a standing seawater system (Boyd *et al.*, 1986) until their use in experiments. Thereafter they were maintained in a running seawater system (Rinkevich and Weissman, 1987a). Two sets of experiments were performed. In the first, larvae were allowed to settle near their parental colonies. In the second, larvae were allowed to settle on maternal-colony tunic.

First set of experiments

Six colonies (= cases) were placed separately, in six 4 l glass tanks just before larvae were hatched. The tanks were aerated by an airstone and maintained in 18°C by a 50W aquarium heater. The glass slide on which each colony had been grown (5 × 7.5 cm) was placed vertically in a slot of a glass staining rack. A blank slide was placed in the nearest slot, facing the sexually matured colony. Hatched larvae settled immediately after their release on the maternal colony tunic, adjacent to the parental colony, or on the blank slide. Larvae metamorphosed to oozoids rapidly thereafter. The slides were transferred to 17 l tanks in a running seawater system two days later. Progeny that settled on the colony tunic were removed and discarded, as were dying or poorly developed zooids that settled on the two glass slides. Several developed

oozooids (3–7 experimental oozooids in each case) were left undisturbed near their parental colony. Under the dissecting microscope other well-developed oozooids were carefully peeled from the blank slides with small pieces of razor blade attached to a firm handle. They were translocated with a Pasteur pipette to other 5×7.5 cm glass slides to serve as controls (20–44 controls per case, total of 207 control offspring). Observations were taken at least once each week, when the mother-colonies and the offspring were cleaned with a soft small paint brush. Experimental offspring that interacted with the colonies were observed 4–5 times a week. All control offspring of a given colony together with their natal colony and the experimental offspring were kept within the same tank under comparable conditions.

Second set of experiments

Five large, older colonies (ages 15–20 months) were used. Each colony had grown over most of its glass-slide surface area. However, at least 30% of the animal surface area was characterized as “dead tunic.” A dead tunic is designated as the tunic of the resorbed, old part of a large colony that does not encompass ampullae and active blood vessels. A dead tunic quite often also appears between systems which spread apart, in a growth pattern distinct from most colonies found in the wild but which often characterizes laboratory-cultured *Botryllus* colonies. Dead tunic frequently includes relics of resorbed zooids, buds and unhatched embryos, and is covered on its upper side by fouling organisms, and on its lower side (facing the glass substrate) by unicellular algae. In this set of experiments we followed the survivorship of offspring settled on dead tunic of their natal colonies. All other offspring settled on the slide, on the mother colony zooids, or near the big colony were removed by a small piece of razor blade.

RESULTS

First set of experiments

Twenty-eight experimental progeny interacted with the six parental colonies (cases I–VI) in the first set of the experiments. Four different types of interactions were observed and designated as: A = fusion, leading to resorption of the offspring; B = fusion, followed later by separation of the progeny and the maternal colony (disconnection); C = tunic-to-tunic contact between the offspring and the parental colony accompanied by the death of the progeny; D = tunic-to-tunic contact, followed by disconnection of the two interacting individuals (Table I). The colony size of each observed experimental progeny and the mean body size of the control progeny for each case are given in Figures 1 and 2. In this series of experiments (Figs. 1–4, Tables I, II) zooids which remained in contact with the natal colony suffered growth failure and poor survivorship, whereas those zooids which subsequently disconnected from the natal colony both survived and grew. These results are detailed below in a case-by-case analysis.

Case I (Table I, Figs. 1, 3a–d). Seven offspring directly contacted with the parental colony. Five of them (nos. 1, 3, 5, 6, 7; Fig. 1) fused or interacted by tunic-to-tunic contact only and thereafter died or were resorbed without growing. In one case (progeny no. 5) the offspring survived for 76 days, whereas oozooid no. 1 resorbed 48 h after fusion was recorded. When oozooid no. 1's body was resorbed, the left ampullae and blood vessels continued to operate for an additional one month while they gradually disintegrated (Fig. 3a–d). Only two colonies (nos. 2, 4) survived: offspring no. 4 remained attached to the dead tunic of the mother colony for 71 days and then sepa-

TABLE I

Summary of interactions between offspring and their parental colonies in the first set of experiments

Case	No. of offspring	Interaction type ^a				Remarks ^b
		A	B	C	D	
I	7	3	1	2	1	B = Disconnected after 20 days as a result of subsequent degeneration of the mother colony. D = The offspring attached to "dead" tunic of the mother colony.
II	5	—	1	4	—	B = Disconnected after 57 days as a result of subsequent degeneration of the mother colony and the offspring.
III	3	—	1	2	—	B = Disconnected after 81 days as a result of subsequent degeneration of the mother colony and the offspring.
IV	3	—	—	3	—	
V	6	2	1	3	—	B = Disconnected after 61 days as a result of subsequent degeneration of the mother colony.
VI	4	1	1	1	1	B = Disconnected after 63 days as a result of subsequent degeneration of the mother colony. D = The offspring attached to "dead" tunic of the mother colony.
Total:	28	6	5	15	2	

^a The interaction type: A = Fusion and resorption of the offspring; B = Fusion and disconnection; C = Tunic-to-tunic contact and death of the offspring; D = Tunic-to-tunic contact and disconnection.

^b "Dead" tunic = A layer of tunic which attached to the colony but does not harbor zooids, blood vessels, or ampulae.

rated, and no. 2 fused with the degenerating part of the parent for 20 days and then separated. This last colony exhibited a significantly higher growth rate than the controls, and on the final day of the experiment (age of 125 days) it possessed 25 zooids (in 2 systems) compared to 6.8 ± 3.4 zooids of the control offspring ($P < 0.001$, *t*-test).

Case II (Table I, Fig. 1). Five offspring attached to their maternal colony tunic. Four of them (nos. 1, 3, 4, 5; Fig. 1) died. Offspring no. 2 remained fused with the mother colony for 57 days and separated as a result of reciprocal degeneration of the two members of the chimera. This progeny continued to degenerate and died 51 days thereafter. Twelve days after the fusion, when progeny no. 2 possessed only four zooids, we observed two large eggs in its buds. We had followed the interactions between this progeny and the parent every other day from the day of fusion and did not observe any development of female or male gonads in the progeny. Five days later, seven large eggs were recorded in progeny no. 2's buds. After 9 additional days and up to the death of this progeny (at the age of 152 days) no more eggs were recorded. The mother colony contained many eggs during the first 14 days after fusion; these disappeared thereafter concomitantly with its own degeneration.

Case III (Table I, Figs. 1, 3e-h). Three offspring attached to the parent tunic. Two (nos. 2, 3; Fig. 1) died without fusion. Offspring no. 1 fused and disconnected 81 days later as a result of reciprocal degeneration (Figs. 1, 3e-h). This progeny continued to degenerate and died 21 days thereafter.

Case IV (Table I, Fig. 2). Three progeny attached to the mother colony tunic. They died 12-47 days thereafter without growth.

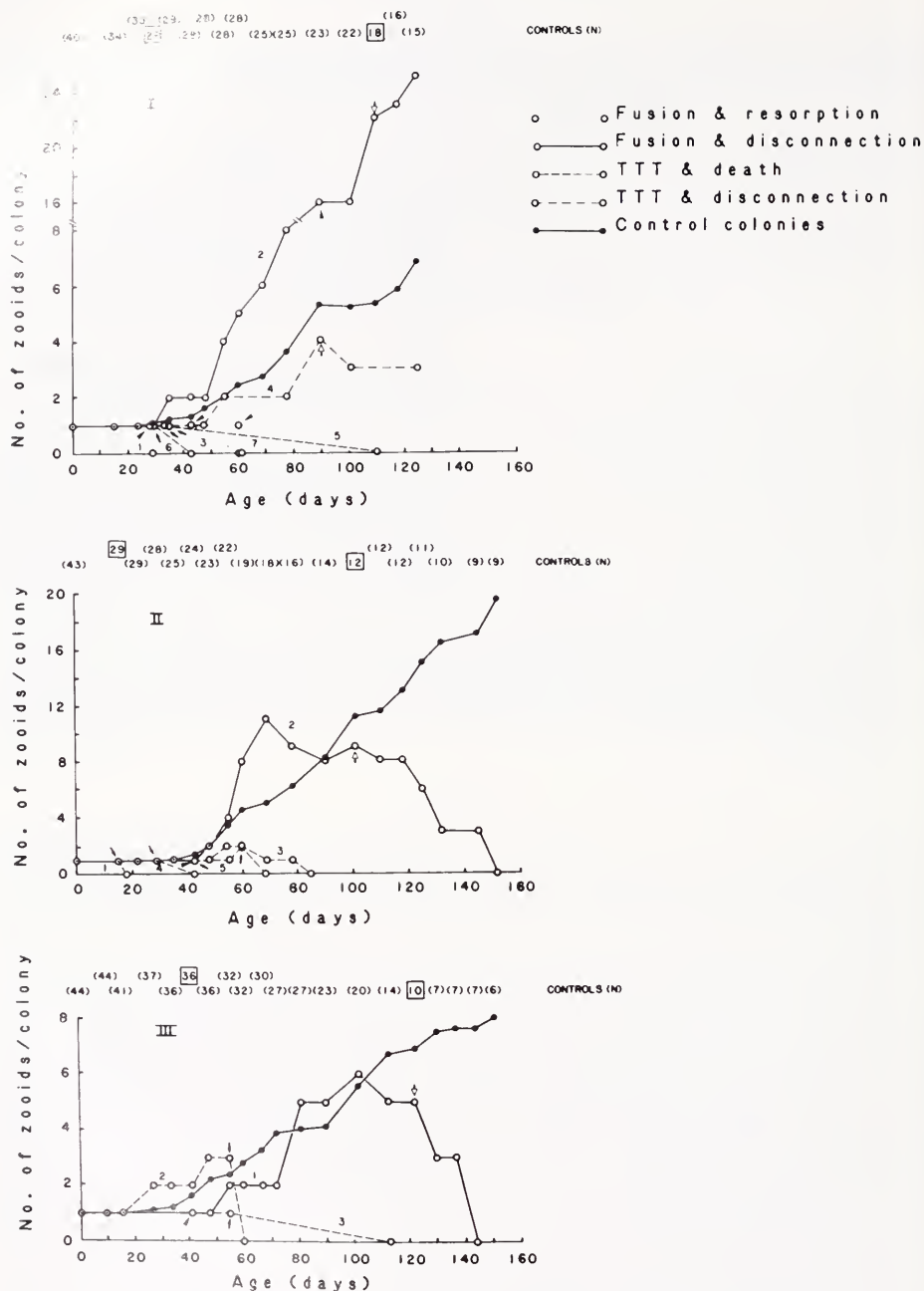


FIGURE 1. *Botryllus schlosseri*. Cosettlement of offspring near their maternal colony. Cases I to III, represented by 7, 5, and 3 experimental offspring, respectively (each experimental offspring is marked by a number). The average body size of the controls is marked by a solid line and black circles. The experimental offspring are marked by open circles. A small black circle within an open one represents a situation in which the body sizes of the controls and the experimental offspring are the same. The numbers in parentheses in the upper part of each case represent the number of surviving controls, while those in boxes provide the number of controls on the day of first offspring-parental interaction and on the day of termination of the last offspring-parental interaction. A black arrowhead = fusion; a black arrow with an open head = disconnection; TTT = tunic-to-tunic contact.

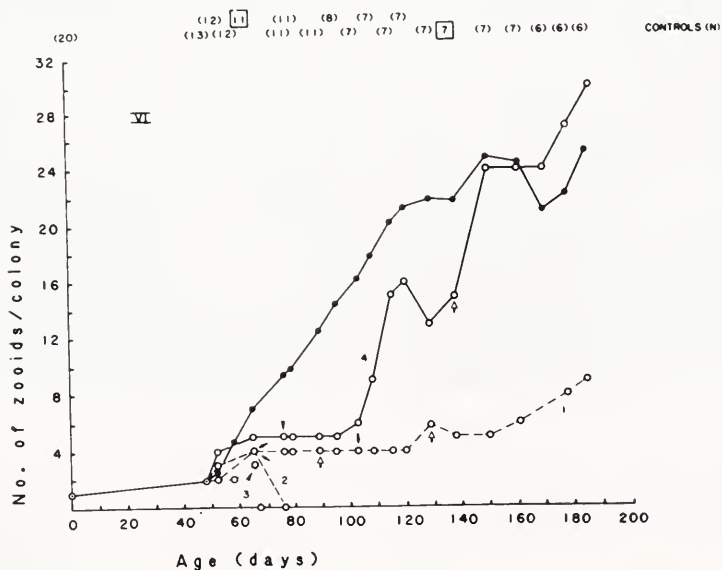
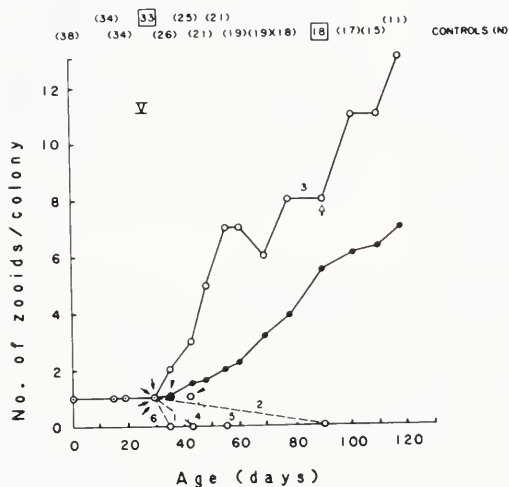
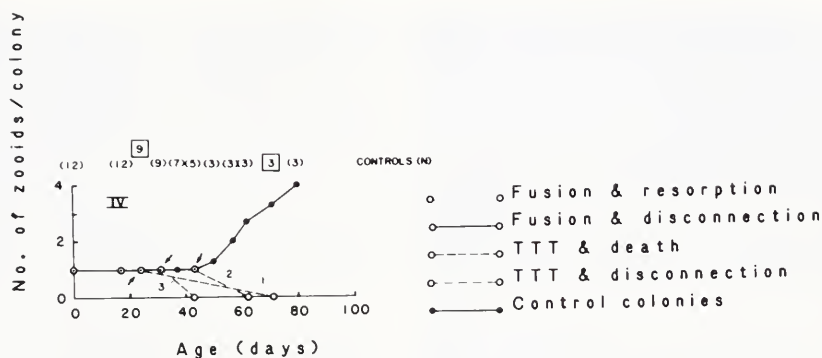


FIGURE 2. *Botryllus schlosseri*. Cosettlement of offspring nearby their maternal colony. Cases IV to VI, represented by 3, 6, and 4 experimental offspring, respectively (each experimental offspring is marked by a number). See legend to Figure 1 for other details.

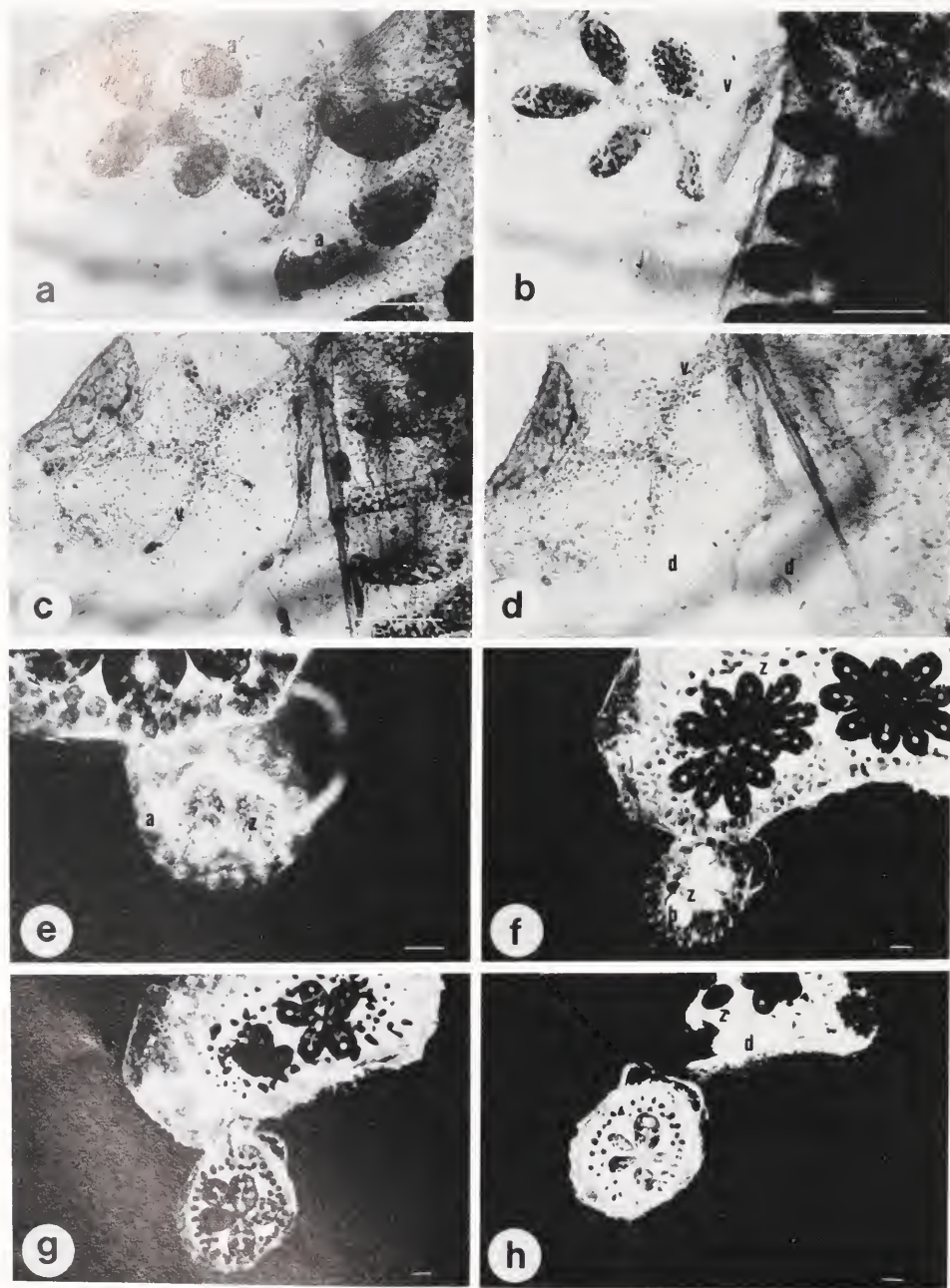


FIGURE 3. Resorption and disconnection of progeny cosettled with their parent. a-d: Case I, offspring no. 1 (refer to Fig. 1). (a) Immediately after resorption, the relict of the resorbed zooid is still seen, with five operating ampullae connected with the parent by one vessel. (b) Six days later, the zooid's body is completely resorbed, while the ampullae are still functional. (c) After an additional eight days, the ampullae are resorbed but the blood vessel still permits blood flow. (d) A month after the zooid's resorption, the blood vessel and ampullae have disintegrated. The dead tunic, overgrown by bacteria, was loosely adherent to the substrate and was detached from the substrate three days later, during a routine cleaning of the

Case V (Table I, Figs. 2, 4a-e). Five of the six offspring that attached tunic-to-tunic or fused with their maternal colony died without growth. A typical fusion-resorption process is documented with offspring no. 4 (Fig. 4a-b). Progeny no. 3 represents another case of egg transfer from the mother colony (Fig. 4c-e). This progeny grew to 2 and 3 zooids 6 and 14 days after fusion, respectively. In this last day (age 43 days) one big egg was observed in a bud (Fig. 4c), but no male gonads were found. The maternal colony contained many similar-size eggs. Progeny no. 3 grew rapidly and 61 days after fusion it disconnected from the parent as a result of degeneration and the death of the mother colony. At the end of the observation period (day 118) progeny no. 3 contained 13 zooids and was significantly larger than the controls (7.0 ± 3.4 zooids; $P < 0.01$, t -test).

Case VI (Table I, Fig. 2). Two (nos. 2,3) of the four offspring which attached to the parent colony died or were resorbed within 11 days. Offspring no. 1 attached to the mother colony tunic for 63 days and disconnected because of degeneration of the old part of the parent. This colony grew significantly slower than the controls, and on the day of separation (age 129 days) it contained only 6 zooids compared to 21.9 ± 9.4 zooids of controls ($P < 0.01$; t -test). Offspring no. 4 fused with the parent and disconnected after 62 days, concomitant with the large colony's degeneration. On the day of separation (age 138 days) this offspring contained 15 zooids (compared to 21.7 ± 9.5 zooids of controls, $P > 0.05$, t -test). Offspring no. 4 multiplied to a 30 zooid colony after an additional period of 47 days.

We calculated the controls' survivorship-percentage in each case by subtracting the number of surviving controls on the day of termination of the last offspring-parental interaction from the number of controls on the day of first offspring-parental interaction, and found 46.3% survivorship (68 out of 147 of the control offspring were alive). By contrast, when we group the offspring which were eliminated by resorption and mortality together, only 7 out of 28 experimental offspring survived (25%). In fact, survivorship eventually was even less: two experimental offspring [case II2 and case III1] were included in the group of survivors although they were already in the process of dying, since according to the pre-determined criterion, they were still alive when re-separated from their parental colonies. The experimental offspring have a significantly higher proportion of "exclusion" than the controls ($P < 0.05$; testing equality of two percentages). However, there is the argument that the resorption of the fused oozoids by their parent-colonies cannot be equated with, and is not equivalent to, the death of those offspring which attached but did not fuse with natal colonies. According to this point of view only 2 out of 17 offspring (11.8%) survived. This mortality rate is significantly higher than that of control offspring ($P < 0.01$; testing equality of two percentages).

animals. e-h: Case III, offspring no. 1 (refer to Fig. 1). (e) Fusion of the offspring with the parent involves one vessel. The offspring possessed two zooids. (f) Seventeen days later, the offspring is a small colony with two zooids and four buds, before changing asexual generations. The ampullae in the parent colony have retreated, and there was no growth of the parent. The nearest two systems to the fused offspring contained 21 zooids. (g) Forty-one days later, degeneration and partial mortality of the parent has occurred, with the two nearest systems to the offspring containing only eight zooids. All ampullae of the parent retreated, exposing a bare, dead tunic. The offspring was still connected by one operating blood vessel to the mother colony. No growth occurred in the offspring colony. (h) Nine days later, resorption and death of most of the maternal colony continued with only four zooids remaining near the offspring. The offspring had disconnected from the mother colony, was in fair condition, but with no growth. a = ampullae, b = bud, d = dead tunic, rz = resorbed zooid, v = blood vessel, z = zooid. Scale bar: a-d = 0.2 mm, e-h = 0.5 mm.

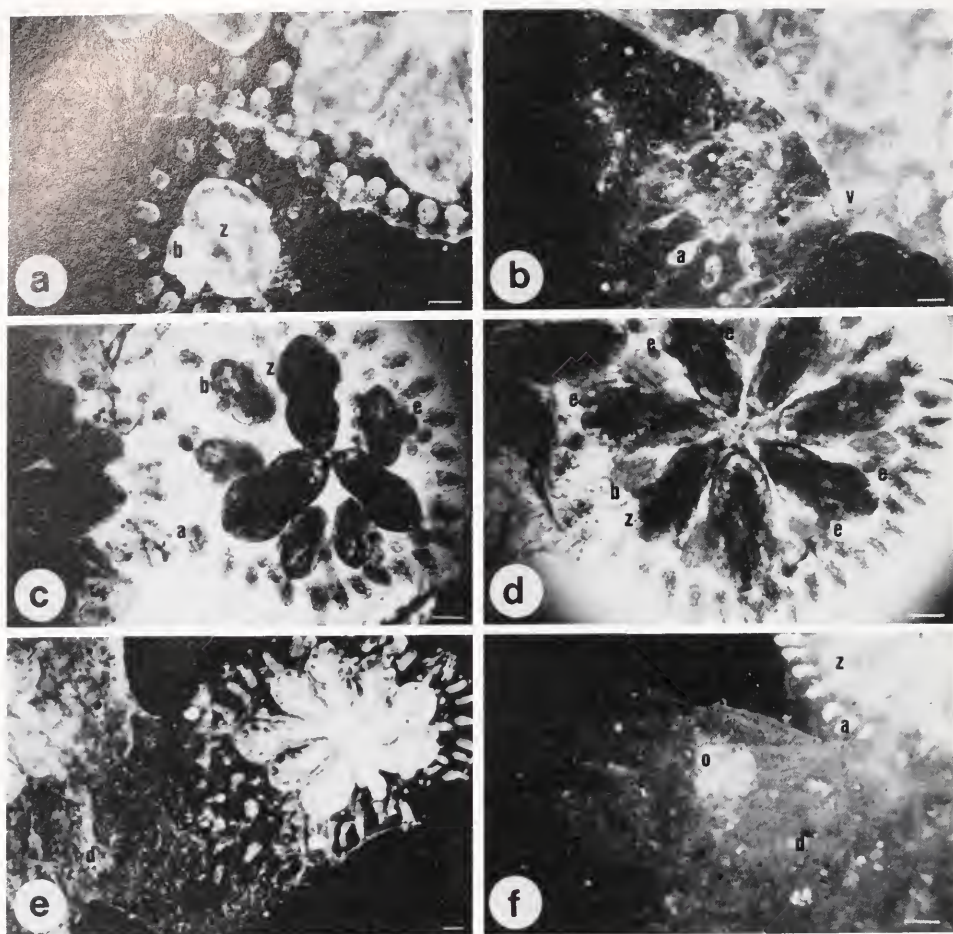


FIGURE 4. Settlement of *Botryllus* larvae near or on the parental colony. a-e: Case V (refer to Fig. 2). a-b: Offspring no. 4, represents a typical resorption. (a) Immediately after fusion. (b) Eight days later, resorption, only ampullae remained. c-e: Offspring no. 3, eggs translocated from the mother colony to the progeny. (c) Fourteen days after fusion, the offspring had three zooids and one egg in a developed bud (picture was taken from underneath the colony). (d) Twelve days later, the offspring colony with seven zooids and seven large eggs (only 5 can easily be recognized) in the developed buds (picture was taken from underneath the colony). (e) Twenty-three days later, shortly before disconnection, the dead tunic was connected between the fused colonies. (f) Second set of experiments. Settlement of oozoid on dead tunic of its maternal colony. a = ampulla, b = bud, d = dead tunic, e = egg, o = oozoid, v = blood vessel, z = zooid. Scale bar = 0.5 mm.

Table II presents some characteristics of the fusion and the tunic-to-tunic interactions between the offspring and parental colonies. The processes which resulted in the elimination of the offspring (resorption and tunic-to-tunic contact followed by a death) lasted a shorter period than the other two processes which resulted in the reparation of the offspring from the parents. Resorption is the fastest process (on the average about one week, Table II) while the two disconnecting processes lasted on average about 2 months. In addition, most of the offspring which ultimately were resorbed and killed during tunic-to-tunic contact did not grow at all (20 out of 21).

By contrast growth was recorded in all the 7 offspring in the other two types of interactions that led to disconnection ($P < 0.01$; Fisher's exact test for independence).

Second set of experiments

The survivorship of 93 offspring settled on the dead tunic of 5 parental colonies was recorded every 2 weeks for a period of 2 months (Table III). The experimental colonies were carefully chosen for their size and the relative large surface area which encompassed dead tunic only (Fig. 4f). Some of the offspring died (natural death, or the effect of the mother colony?). However, most of them were killed by the lifting of the degenerated parental colony tunic from the substrate. Since the offspring were attached to the tunic and not to the substrate, they were swept out from the slide and died. After one month only 18.3% of the offspring survived. Two months after settlement no offspring survived (Table III).

DISCUSSION

Co-settlement of *Botryllus* progeny adjacent to their maternal colony (first set of experiments) or on their mother colony's dead tunic (second set of experiments) clearly resulted in increased offspring mortality. Most of the results in this study were obtained within 2 months of the first tunic-to-tunic contact. Resorption was the fastest interaction recorded (about 1 week) and is much faster than the resorption obtained when two large colonies or two big subclones of different colonies fused (up to 8 months after fusion; Rinkevich and Weissman, 1987a). The fast resorption recorded in the present study could be the result of the huge body-size differences between the parent and the offspring. The colony body size was found to be an important factor for determination as to which colony in a chimera will be resorbed (Rinkevich and Weissman, 1987a). Only 3 out of 11 (27.3%) progeny survived the resorption and the separation from the parent (Figs. 1, 2; Table II). This number is too low to support the proposal that fusion of closely related genotypes on the histocompatibility locus is always beneficial. In fact, the results of the present study indicate that the survivorship of progeny fused with a parent is possible only when associated with the degeneration or mortality of the parental colony (Table I).

Another interesting result in the first set of experiments is that most of the offspring (60.7%) did not fuse with their maternal colonies, although they share alleles in common with the parents in the fusibility locus. Part of this is due to offspring settlement near an "old" part of the parent which did not possess active ampullae. Active, healthy ampullae of growing parts are vital for a successful fusion between compatible colonies (Taneda, 1985; Y. Saito, pers. comm.). However, in other cases, although offspring and parents confronted active, good ampullae, they did not fuse for a long time (up to 2 months); thereafter the offspring died. Out of the 17 tunic-to-tunic interacting pairs only 2 progeny survived: those that re-separated from their parental colony (Table II). In addition, these two offspring (cases I4, VII1; Figs. 1, 2) grew significantly slower than the controls. These results may indicate a possible role of humoral factors and/or cellular elements present in the tunic of intraspecifically interacting *Botryllus* colonies. Diffusing of allogeneic humoral factors through the text matrix was shown to exist in at least two species of botrylloid ascidians, *B. primigenus* and *Botrylloides simodensis* (reviewed by Taneda *et al.*, 1985).

In two cases mature eggs were observed within the offspring soon after fusion with their parents. The parental colonies contained at the same time many similar eggs. In contrast, none of the control progeny exhibited any sign of sexual development.

TABLE II
Characteristics of fusion and tunic-to-tunic contacts between offspring and their maternal colonies in the first set of experiments

Fusion, resulted in offspring				Tunic-to-tunic, resulted in offspring					
Resorption				Disconnection			Death		
Off-spring ^a no.	Size ^b (zooids)	Time ^c scale (days)	Growth during inter-action	Off-spring no.	Size ^b (zooids)	Time ^c scale (days)	Off-spring ^a no.	Size ^b (zooids)	Growth during inter-action
I 1	1	1	—	I 2	16	20	I 5	1	75
I 3	1	17	—	II 2	1	57	I 6	1	14
I 7	1	1	—	III 1	1	81	II 1	1	3
V 4	1	8	—	V 3	1	61	II 3	1	42
V 5	1	12	—	V 14	5	62	II 4	1	15
V 13	3	2	—				II 5	2	9
							III 2	3	5
							III 3	1	58
							IV 1	1	47
							IV 2	1	18
							IV 3	1	12
							V 1	1	14
							V 2	1	61
							V 6	1	6
							V 12	4	11
X ± SD (n)	6.8 ± 6.1 (6)				56.2 ± 19.9 (5)			26.0 ± 22.9 (15)	
									67.0 ± 4.0 (2)

^a The offspring numbers refer to the colony case (I to VI); Figures 1 and 2.

^b Size in the day of fusion or colony-to-colony contact.

^c Time scale: the length of the interaction.

TABLE III

Survivship of offspring settled on "dead tunic" of their maternal colonies

Colony no.	No. of settled offspring	No. of offspring after			
		2 weeks	4 weeks	6 weeks	8 weeks
1	25	15	3	0	0
2	18	16	2	0	0
3	12	10	1	0	0
4	22	18	6	2	0
5	16	13	5	1	0
Total	93	72	17	3	0

Egg development in *Botryllus* differs from any other ordinary case of protogyny in that the first developed young ova migrate in the blood stream to the second generation of buds developed from the existing generation of zooids. These ova continue to develop, although they still do not reach maturity. Rather, they migrate again into the next generation of blastozooids. It is not until the seventh or eighth generation that ova are fertilized (Herdman, 1925). Since no such egg development was observed in the fused progeny nor the typical simultaneous male gonad development (Mukai, 1977; Sabbadin and Zaniolo, 1979) the observed large eggs in the fused progeny likely migrated from the mother colony through the connecting blood vessels. Germ cell exchange between fused *Botryllus* colonies has been recorded before (Sabbadin and Zaniolo, 1979). In some hatches of offspring, the progeny released from fused colonies were mostly or totally heterochthonous (Sabbadin, 1982). This suggests the possibility of germ cell parasitism (discussed by Buss, 1982), in addition to the resorption which is related to somatic cell parasitism (Rinkevich and Weissman, 1987a,b). Another pattern of parasitism, oriented translocations of materials from the inferior colony towards the superior member within a chimera, was recorded in fused colonies of the hermatypic coral *Stylophora pistillata* from the Red Sea (Rinkevich and Loya, 1983). This phenomenon was coupled with a significant reduction of growth rate and reproduction of the inferior colony (Rinkevich and Loya, 1985). These findings suggest a possible complex network of physiological interactions occurring after a fusion between two compatible colonies is established. Unfortunately, in these experiments the potential germline chimerism, or parasitism, could not be tested critically, as fertilization of the eggs did not occur. Thus we can neither affirm that the eggs were of parental origin nor that they might have enjoyed a selective reproductive advantage.

Grosberg and Quinn (1986) postulated that *Botryllus schlosseri* larvae recognize kin on the basis of shared alleles at the histocompatibility locus and that this recognition promotes cosettlement of histocompatible individuals. They suggested that fusion which subsequently evolved from cosettlement of the closely related genotypes is beneficial among kin in several ways, as initially proposed by Buss (1982). One of these benefits is that fusion may increase the probability of survivorship and growth. In contrast, the present paper provides evidence (Figs. 1, 2, 3, 4; Tables I, II, III) that the settlement of offspring very near or on their parental colony may in fact reduce survivorship and growth rates. Similar results of reduction in survivorship and growth rates were obtained in other experiments at Hopkins Marine Station in which pairs of large colonies (Rinkevich and Weissman, 1987a,b) or pairs of sibling oozooids (unpub.) were cosettled.

A number of theories have been proposed that the ability to recognize one's kin is beneficial (reviewed by Hepper, 1986). It is a fact that sibling larvae of *Botryllus* settled in aggregations (Grosberg and Quinn, 1986; this paper) and near their parental colony (Sabbadin, 1978; this paper). However, by keeping in mind the deleterious effects of the fusion, how does one explain the existence of cosettlement of closely related individuals such as in the case of *Botryllus*? One possible explanation considers the selective pressure of other biotic interactions, such as interspecific competition.

Botryllus colonies in the field compete for the substrate with other sessile organisms. For example, *B. schlosseri* from Woods Hole is usually overgrown and killed by *Botrylloides* (Grosberg, 1982). In addition, this competition also mediates the relative frequency of different types of life history tactics presented by *Botryllus* colonies (Grosberg, 1982). In Monterey, California, even large *Botryllus* colonies are often overgrown by *Diplosoma*, a fast-growing colonial tunicate (unpub.).

Buss (1981) has shown that the formation of aggregations in the colonial bryozoan *Bugula turrita* is a response to interspecific competition. *B. turrita* also suffers significant intraspecific competition. However, in high densities *B. turrita* is rarely overgrown or killed by interspecific competition (Buss, 1981). The costs incurred by increased intraspecific competition involve a loss of proliferative potential, whereas the benefits accrued by reduced interspecific competition involve whole-colony mortality (Buss, 1981). Since mortality results in a greater loss in fitness than does a reduction in growth rate, the evolutionary selection for the gregarious settlement is plausible. If the persistence of dense assemblages is necessary in sedentary marine animals for excluding possible interspecific competitors (Jackson, 1983), our results could reflect a general pattern of habitat selection in sessile marine invertebrates which aggregate in settlement *versus* those that do not (Knight-Jones and Moyse, 1961; Crisp, 1979; Buss, 1981; Jackson, 1983; and literature therein).

Working on another colonial bryozoan, Keough (1984) demonstrated that larvae of *Bugula neritina* settle preferentially near conspecific larvae. This observation, coupled with the observation that isolated juveniles of this species did not reach adult size, was attributed to the selective effects of predation by fishes. Fishes rarely took more than five bites on any one feeding visit, so groups of colonies might only be damaged at the periphery. A strong advantage to gregariousness resulted since central colonies in a group were left untouched. There is no record of fusion between *Bugula* colonies, although self/nonsel recognition has been described in bryozoans (Keough, 1984).

In *Botryllus*, on the other hand, one common allele at the fusibility locus is sufficient for fusion between colonies (Scofield *et al.*, 1982). Keeping in mind the two studies on gregarious bryozoans (Buss, 1981; Keough, 1984), we suggest that fusion of cosettled compatible *Botryllus* colonies may have long-term deleterious consequences and may not be functionally beneficial to the chimera *per se*, as suggested by Grosberg and Quinn (1986). We propose that the phenomena of resorption and/or death of interacting conspecific colonies may have evolved as a necessary consequence of the self-recognition phenomenon in *Botryllus*, which allows two different genotypes sharing in common only one allele at the fusibility locus to fuse and form a chimera. More specifically, we suggest that the gregarious settlement of *Botryllus* larvae in the proximity of the parental colonies and the nature of self/nonsel recognition of this species may have initially evolved as two independent phenomena (or strategies) which are in the process of adaptation. As a result, the cost/benefit outcomes depend on the view of the observer. The concept for the general costs of fusion was first presented by Buss (1982) and further discussed in Rinkevich and Weissman

(1987b). It is not the purpose of the present paper to discuss again the costs for the chimera in details. However, following our previous discussion, in our view chimera formation in *Botryllus* could develop only if the evolutionary benefits of cosettlement exceeded the disadvantage of the harmful consequences of fusion. Interspecific competition between *Botryllus* and other sessile organisms (Grosberg, 1982) may provide this very selective pressure (Buss, 1981). Gregarious settlement of *Botryllus* larvae, even with the loss of many progeny (as the results of resorption and/or death), would be evolutionarily selected if the number of survivors in gregarious settlement would exceed the number of survivors of settlement in a random distribution. Perhaps the resorption and death of one of the two colonies in parabiotic union allows the unit of reproductive selection to be the survivor of both intra- and interspecific competition. If so, it shall be most important to define the number and character of genes involved in the self/nonself recognition events leading to death and/or resorption of contacting *Botryllus* kin. Therefore the ecological advantages of gregarious settlement in *Botryllus* and the role of interspecific competition must be addressed in further field and laboratory experiments.

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VARIABILITY IN FLASH CHARACTERISTICS OF A BIOLUMINESCENT COPEPOD

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ABSTRACT

Bioluminescence of the copepod, *Pleuromamma xiphias*, was investigated with an optical multichannel analyzer (OMA) to measure emission spectra, an integrating sphere-photon counting detector system to determine flash kinetics and quantum emission, and an ISIT video system to image spatial patterns of emission.

Light emission was in the blue spectral region, with maximum emission at approximately 492 nm. Spectral waveforms were unimodal, or bimodal with the secondary peak at 472 nm.

Flashes in response to a single stimulus consisted of two components: a fast component attaining maximum intensity in under 100 ms, and a slow element which peaked after 600 ms. The fast component originated from thoracic and abdominal light organs while the slow component represented a large expulsion of luminescent material from the abdominal organ only. Both components exhibited first order exponential decay although the decay rate of the fast component was approximately one order of magnitude greater. The typical flash response to a single stimulus exhibited a response latency of 30 ms, initial rise time of 87 ms, duration of 2.4 s, and quantum emission of 1.4×10^{10} photons flash⁻¹. Quantum emission increased with increasing stimulus strength.

Both response waveform and total quantum emission were affected by the frequency of electrical stimuli. Stimulation at 1 Hz generated the greatest luminescence, averaging 1.1×10^{11} photons response⁻¹ for 11 s emissions. Higher rates of stimulation decreased total quantum emission and response episode duration, and resulted in greater temporal summation of the emission waveform.

Variability in flash characteristics due to electrical stimulation suggests a versatility of luminescent displays *in situ*.

INTRODUCTION

Recent bathyphotometer measurements in the Sargasso Sea suggest that most stimuable bioluminescence in epipelagic waters originates from zooplankton, principally crustaceans such as euphausiids, ostracods, and copepods, as well as other organisms such as larvaceans and radiolaria (Swift *et al.*, 1983, 1985). Copepods represent approximately 70% of the zooplankton specimens in the upper 200 m of the Sargasso Sea. Calanoid copepods are more abundant than other copepods (Deevey, 1971) and include many luminescent species (Herring, 1978, 1985).

The secreted bioluminescence of copepods originates from multiple glands (Giesbrecht, 1895; Clarke *et al.*, 1962), and presumably functions as part of an escape

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Abbreviations: OMA—optical multichannel analyzer, MCA—multichannel analyzer, S/N—signal to noise ratio, FWHM—full width at half maximum amplitude, PMT—photomultiplier tube.

response from predators (David and Conover, 1961; Buck, 1978; Young, 1983). Flashes can be induced by mechanical, electrical, photic, or vacuum (presumably acting as mechanical) stimulation, as well as by the presence of potential predators (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972; Lapota and Losee 1984; Herring, 1985; Yevstigneyev, 1985). After a brief latency following the stimulus, copepods respond with a flash that rapidly rises to maximum intensity and lasts from less than 1 s to more than 10 s.

A striking aspect of copepod bioluminescence is the variability in flash waveforms and kinetics (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972). This makes it difficult to identify trends in flash responses due to experimental manipulations or other factors. Only in coastal ostracods (Morin and Bermingham, 1980; Morin, 1986) has the variability in flash responses been correlated with different luminescent behaviors.

Our study examines variability in the spectral and temporal characteristics of bioluminescence of the calanoid copepod, *Pleuromamma xiphias*. Members of the genus *Pleuromamma* are numerous throughout the year in the Sargasso Sea (Deevey, 1971) and at times it is one of the dominant genera (Fish, 1954). They are active vertical migrators (Roehr and Moore, 1965), ascending from daytime depths of 350 m to epipelagic depths at night where they contribute to measured bioluminescence (Swift *et al.*, 1983, 1985). The flash of *P. xiphias* is similar to that of other members of the Metridiidae and is readily elicited by electrical pulses (Clarke *et al.*, 1962; Yevstigneyev, 1985). Our results indicate that the nature of the electrical stimulus dictates the type of flash response observed, and suggest that complex neural or other factors play significant roles in regulating the kinetics and quantum emission of the flash response.

MATERIALS AND METHODS

Adult specimens of *Pleuromamma xiphias* (Giesbrecht, 1889) were collected and studied during the April, 1985, Biowatt cruise aboard the RV Knorr, and during a subsequent cruise on the RV Endeavor in May, 1987, from stations between 28° and 35° N 70° W in the Sargasso Sea. Plankton nets with 333 μm mesh and 0.5 or 1 m mouth diameters were towed at night for 20 min at depths of 80–100 m. Seawater temperatures at these depths ranged from 18–22°C (T. Dickey, pers. comm.). Specimens were sorted and maintained in filtered seawater in darkness until use. All experiments were performed at room temperature ($22 \pm 2^\circ\text{C}$) within 10 h of collection. Subsequently, specimens were individually preserved in 4% formalin for later identification.

Spectral measurements

Bioluminescence emission spectra were measured with a Princeton Applied Research optical multichannel analyzer (OMA) system. The OMA detector (EG&G PARC Model 1420) consists of a linear array of 700 intensified photodiodes which simultaneously collects the light signal across a 350 nm spectral window of a polychromator. The OMA system has the requisite high sensitivity, high resolution, and fast response time necessary for registering dim, brief bioluminescent emissions. Details of OMA operation and calibrations have been previously described (Widder *et al.*, 1983).

Specimens were suspended in a drop of filtered seawater between a pair of tungsten electrodes and stimulated at 20 Hz for approximately 4 s with 10 V, 5 ms duration monophasic pulses from a Grass model S44 stimulator. Bioluminescence was

focused onto a 1 mm entrance slit to the polychromator by quartz optics and was integrated over a period of 1–2 s by the OMA system. Spectra with signal to noise ratios (S/N) less than 30 were not used (Widder *et al.*, 1983).

Measurements of flash kinetics and quantal output

The temporal characteristics of bioluminescence were measured with an integrating sphere–photon counting detection apparatus. An integrating sphere is considered critical to precise measurements of quantum emission from sources that may not emit isotropically, *e.g.*, most organisms with photophores. Single specimens were suspended in a drop of filtered seawater between a pair of tungsten electrodes while enclosed in a 10 inch diameter integrating sphere (Labsphere, Inc.). The inside surface of the sphere is coated with white Polane polyethylene paint to ensure maximum reflectance (97% reflectance at 500 nm) and minimize damage to the reflector surface from contact with seawater. A baffle between the source and detector assured that only light that had undergone multiple reflections within the sphere was measured. Bioluminescence was detected by a photon counting RCA No. 8850 photomultiplier tube, operating at –1700 V with a calibrated discriminator setting of –0.315 V, that viewed the interior of the sphere through a 4 cm diameter port. The entire apparatus was calibrated for photon emission both before and after the cruise with an Optronic Laboratory model 310 calibration source referenced to an NBS standard. Quantum calibration took into account not only the spectral responsivity of the integrating sphere and photomultiplier tube but also the spectral emission of bioluminescence of *P. xiphius* as measured by the OMA. The calibration of the system was frequently checked at sea with a C^{14} phosphor (I-Lite, 0.05 mCi) referenced to the Optronics source.

Bioluminescence was stimulated by single or repetitive electrical pulses at various frequencies, while the photomultiplier signal was monitored for either 8 or 20 s with an Ortec No. 776 counter/timer and a Norland No. 5400 multichannel analyzer (MCA). Flash waveforms displayed on the MCA were either directly photographed or videotaped. Printed copies of the flash waveforms were later obtained from the video record after processing by a Megavision model 1024XM image analysis system. Flash kinetics were derived from the printed waveform on a Summagraphics digitizing pad.

Flash characteristics are defined as follows: response latency = time from presentation of the stimulus to beginning of the response; total rise time = time from beginning of the response to maximum intensity of emission; 50% decay time = time from maximum intensity to an intensity one-half that value; total flash or response duration = time from beginning to end of response; response episode = light emission during repetitive stimulation; (total) quantum emission = total integrated photons of response episode; maximum response = response with greatest total integrated photons.

Image intensification

Individual specimens were placed between two metal electrodes in a clear leucite chamber containing approximately 2 ml of filtered seawater. The chamber was enclosed in a sealed box with white reflective internal surfaces. Bioluminescence was viewed from above through a port in the box with an intensified SIT video camera (Dage-MTI Model 66) fitted with a 105 mm Nikon F/4.0 lens. A photon counting detection system (described above) obtained simultaneous measurements of flash waveforms and kinetics. The photon counting tube viewed the interior of the box

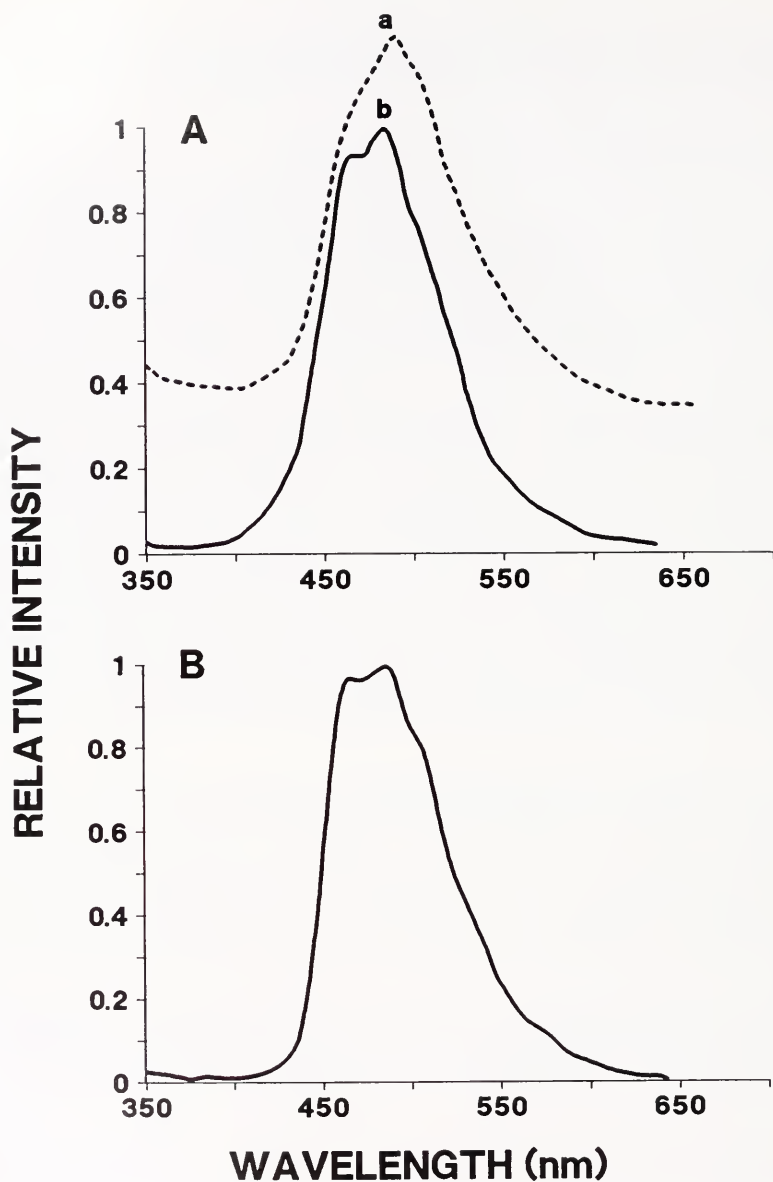


FIGURE 1. Mean emission spectra of the bioluminescence of *Pleuromamma*. (A) *Pleuromamma xiphias* spectra are displayed on the same intensity scale but are vertically displaced for clarity. (a) Unimodal spectral distribution (dashed line) representing emissions from three specimens; max = 493 nm, FWHM = 83 nm, S/N = 103. (b) Bimodal spectral distribution (solid line) from four specimens; max = 492, 472 nm, FWHM = 74 nm, S/N = 97. (B) Spectral distribution representing two specimens of *P. abdominalis*; max = 486, 465 nm, FWHM = 75 nm, S/N = 70.

through a 4 cm diameter port, and measured only reflected bioluminescence. Quantum calibration of this system was not performed.

Light production was stimulated electrically by single or repetitive pulses. The

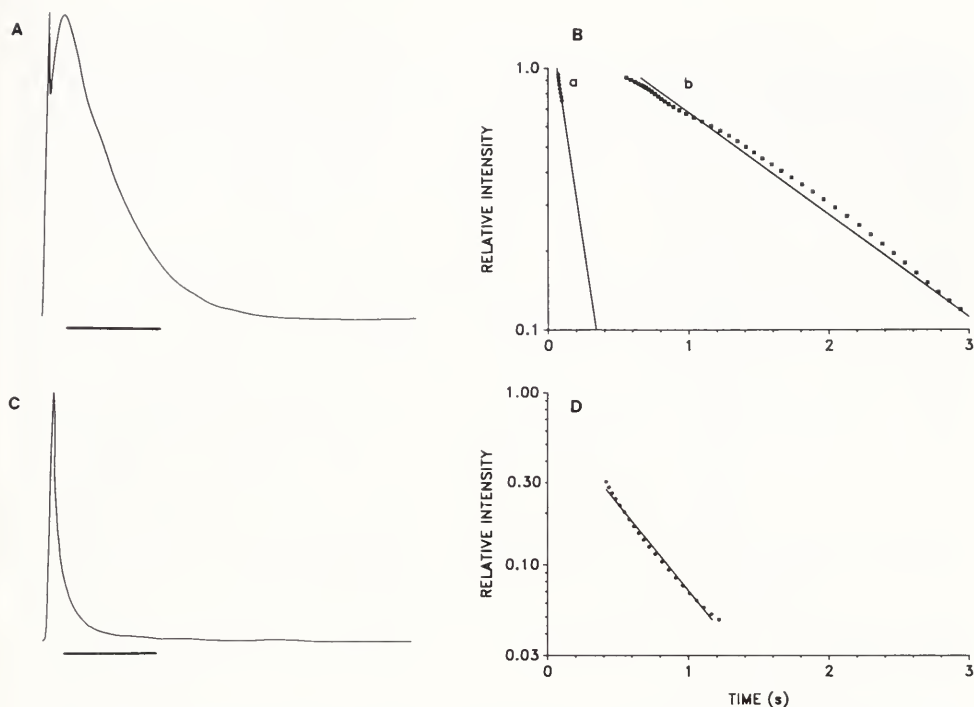


FIGURE 2. Luminescent responses of two specimens of *Pleuromamma xiphias* to a single 10 V, 5 ms duration electrical stimulus. (A) Typical flash response exhibiting fast and slow components. The relative intensity of emission is displayed with time; time bar = 1 s. (B) Decay kinetics of both components of the flash displayed in (A). Relative intensity (log scale) is shown as a function of time. For each component the slope of the calculated linear regression (solid lines) reflects the rate of exponential decay (refer to text). Decay rate of the fast component (a) was -8.3 while the decay rate of the second component (b) was -0.9 ($R = 0.99$ for each). (C) Flash lacking the second component. As in (A) except that the vertical scale is magnified 10 times. (D) Decay of light emission of the flash displayed in (C). As in (B). The decay rate was -2.3 ($R = 0.99$).

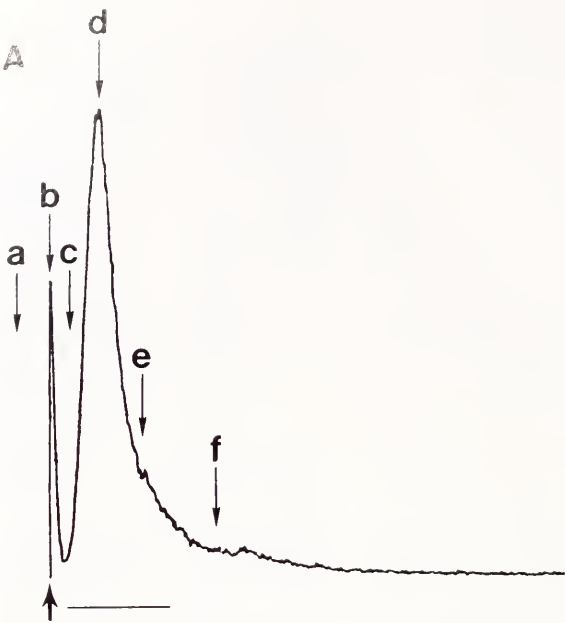
methods of collection and analysis of data were identical to those described in the previous section.

RESULTS

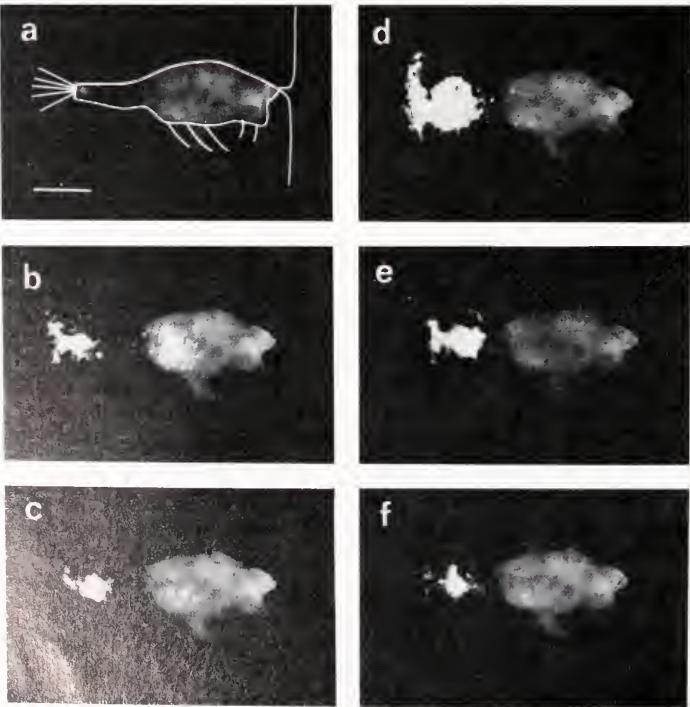
Spectral characteristics

Bioluminescence emission spectra from 7 specimens of *Pleuromamma xiphias* were centered in the blue region of the visible spectrum with maxima at approximately 492 nm. Two types of spectral distributions were measured; about half of the specimens produced unimodal spectra while the others generated bimodal spectra (Fig. 1). Regardless of the spectral shape, the dominant emission was at 492–493 nm while the short-wavelength 472 component was present either as a subpeak or shoulder.

Bimodal emission spectra were also measured from the two specimens of *P. abdominalis* tested. They differed only slightly from those of *P. xiphias*, having maximal emission at 486 nm and a short-wavelength subpeak at 465 nm (Fig. 1). Neither species gave evidence of sexual differences in spectral emissions.



B



Temporal characteristics and quantum emission

Flashes from approximately 350 specimens of *Pleuromamma xiphius* were analyzed for kinetics and quantum emission. No spontaneous flashes were observed from specimens in the apparatus prior to testing, although luminescence often was elicited by handling during preparation. Even though there was much variability in the kinetics and quantum emission of luminescent responses, it was possible to identify general trends in responsiveness.

The waveform of a flash response to a single electrical pulse (Fig. 2A) was resolved into two components. The first, characterized by fast rise and decay times, was followed, after a slight decay in intensity, by a second element consisting of a slower rise and decay (see Fig. 5B for expanded waveform). The maximum intensity of the second peak was equal to or greater than the first peak. The majority (25 out of 46) of flashes stimulated by single electrical pulses contained both components. The remaining flashes were composed of either the fast or slow component only (7 and 6 flashes, resp.), or had a waveform that was not possible to resolve (8 flashes). Quantum emissions of flashes with only one component (Fig. 2C) were from one to two orders of magnitude lower than those of two-component flashes.

Preliminary observations indicated that mechanical stimulation of light emission also elicited flashes with fast and slow components, although the kinetics and quantum emission of mechanically stimulated flashes were not investigated.

Image intensification of single specimens during the production of two-component flashes revealed the spatial pattern of emission (Fig. 3). The fast component was typically produced by a brief emission from a luminescent gland located laterally on thoracic segment 3 or 4, and simultaneous light production from another light organ located on the caudal rami (Fig. 3b). The luminescent material was not violently expelled from the body during this fast component. The decrease in light intensity following the fast component resulted from a cessation of thoracic light organ production along with a slightly diminished emission from the abdominal light organ (Fig. 3c). The slow flash component was due to increased production of luminescent material by the abdominal light organ and subsequent expulsion from the body (Fig. 3d). No other light organs were observed to be active at this time. The decay of the slow component was due to a gradual decrease in the amount of luminescent material produced by the abdominal light organ (Fig. 3e, f).

Flashes in response to a single 10 V stimulus (Table I) had a mean stimulus-response latency of 18 ms. The initial peak of the fast component occurred within 100 ms while the slower second component reached maximal intensity approximately 600 ms after the stimulus presentation. Total flash duration was 2.6 s, and the average quantum emission of a single flash was 1.4×10^{10} photons flash⁻¹ (maximum of 7.1×10^{10} photons).

Decay of light emission was measured for each component of two-component flashes ($n = 7$). Bioluminescence decreased exponentially with time according to the

FIGURE 3. Luminescent response of a single specimen of *Pleuromamma xiphius* to a single 40 V, 5 ms electrical stimulus monitored by simultaneous image intensification and photomultiplier recording. (A) Flash waveform from the MCA showing fast and slow components. Intensity is shown as a function of time. Time bar = 2 s. (B) Simultaneous images of animal (lateral view) obtained from single frames of the video record. Scale bar = 1 mm. Letters (a) through (f) in both portions of the figure correspond to identical time periods during the flash: (a) prior to stimulation; (b) maximum emission during fast component; (c) decay in intensity of fast component; (d) maximum emission during slow component; (e) and (f) decay of slow component.

TABLE I

*Response kinetics and quantum emission of flashes of *Pleuromamma xiphius* stimulated with single electrical pulses (10 V, 5 ms duration)*

Latency (ms)	Total rise time (ms)		50% decay time (s)	Total flash duration (s)	Quantum emission (photons flash ⁻¹)
	Fast component	Slow component			
18.4*	87.1	603.0**	0.9	2.6	1.4×10^{10}
± 4.2	± 15.8	± 254.7	± 0.3	± 0.7	$\pm 0.6 \times 10^{10}$
(0.5–59)	(27–202)	(552–838)	(0.02–2.8)	(0.1–10)	(6.1×10^7 – 7.1×10^{10})

* Values represent the mean values $\pm$ standard errors of the mean, with ranges in parenthesis, for 14 specimens.

** Precise measurements of second component kinetics were possible in only three specimens.

standard equation for exponential decay, $Y = A * e^{(B * t)}$, where A is the y-intercept and B is the decay rate constant. Decay rates were calculated from the linear regressions of the natural logarithm of intensity with time (Fig. 2B, 2D; $R = 0.99$ for all regressions). The mean ($\pm$ S.E.) rate of decay of the fast component of -14.6 ± 4.4 was an order of magnitude greater than the rate of decay of the second component (-1.25 ± 0.5).

A separate experiment demonstrated that flash quantum emission increased as a function of stimulus strength when animals were tested with single stimuli of 5 ms duration from 2–10 V in magnitude (Fig. 4). Minimum responses were at 2 and 4 V, where the average emission was approximately 3.8×10^9 photons flash⁻¹. Threshold

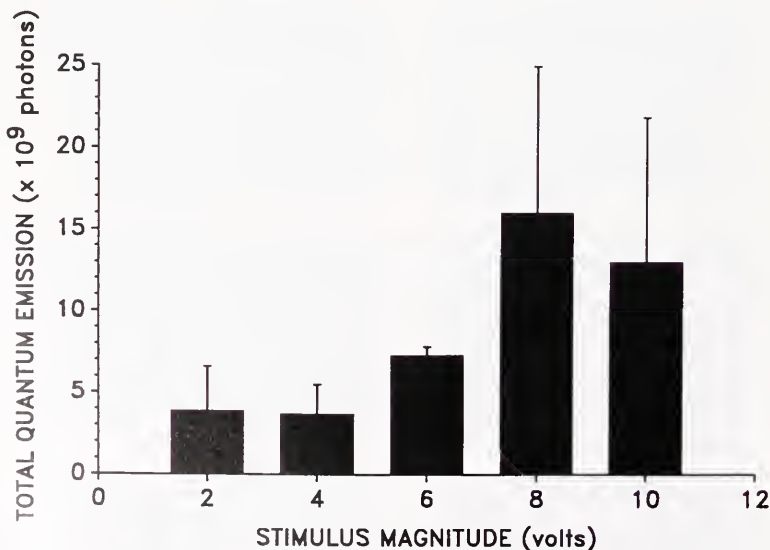


FIGURE 4. Quantum emission of *Pleuromamma xiphius* flashes as a function of the voltage of single 5 ms duration electrical pulses. For each stimulus condition, 6–7 specimens were tested. Mean values ($\pm$ standard errors) are: 2 V, $3.9 \pm 2.7 \times 10^9$ photons; 4 V, $3.7 \pm 1.8 \times 10^9$ photons; 6 V, $7.3 \pm 0.5 \times 10^9$ photons; 8 V, $1.6 \pm 0.9 \times 10^{10}$ photons; 10 V, $1.3 \pm 0.9 \times 10^{10}$ photons. Due to the large standard errors, mean values were not significantly different from one another (t -test, $P > 0.05$), although maximum flash quantum emission at each stimulus voltage level increased.

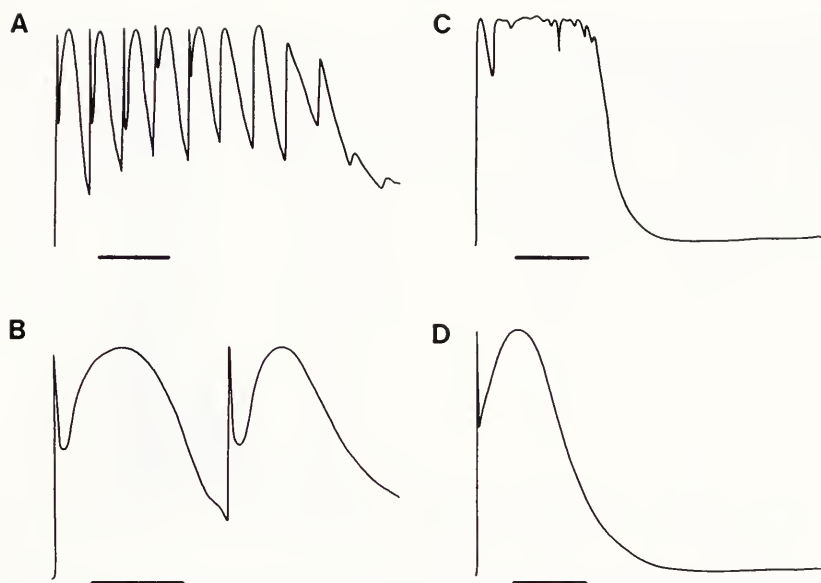


FIGURE 5. Luminescent responses of *Pleuromamma xiphius* as a function of repetitive stimulation with 10 V, 5 ms duration electrical pulses. Intensity of output (same relative scale) is displayed as a function of time. Time bars = 2 s except in (B). The maximum intensity of the responses was near the threshold of non-linearity of the detection system and may underestimate true intensity levels. (A) Response episode of one specimen to 0.5 Hz stimulation. (B) Detail of individual flashes comprising response episode for 0.5 Hz stimulation; fast and slow components of each flash are evident. Time bar = 1 s. (C) Response to 2 Hz stimulation; temporal summation produces a single prolonged response episode. (D) Response to 10 Hz stimulation. The response is similar in waveform to that for single pulse stimulation but of longer duration.

was apparently near 2 V, since at that level only 50% of the specimens tested produced luminescence, while at higher voltages all stimuli elicited a response. Stimulus voltage had no effect (Student's *t*-test, $P > 0.05$) on response latency (mean and standard error = 36 ± 7 ms), initial rise time (123 ± 21 ms), 50% decay time (856 ± 201 ms), or flash duration (2.8 ± 0.5 s). These values are not significantly different from those of the previous experiment with a constant 10 V stimulus (Student's *t*-test, $P > 0.05$).

Stimulation with repetitive pulses had the most pronounced effect on response waveform and quantum emission (Fig. 5). At the slowest stimulus rate of 0.5 Hz (Fig. 5A), the response episode consisted of individual flashes separated by partial decay of light intensity. There was a 1:1 stimulus-response correlation and no temporal summation or facilitation of single flashes. In many instances luminescence was not exhausted during the 20 s data collection period, which was equivalent to 11 stimuli; therefore the measured quantum emission underestimated the total stimutable emission. The waveform and kinetics of single flashes comprising the response episode were similar to those for single pulse stimulation; in fact it was usually possible to resolve the fast and slow flash components (Fig. 5B).

When stimulated at 1 and 2 Hz, components of individual flash responses to each stimulus pulse were still evident, but were temporally summated to form a single prolonged emission with an average duration of approximately 8 s (Fig. 5C). At 1 Hz stimulation, the average total duration of the response episode was approximately 14 s and the mean total emission was 1.1×10^{11} photons episode⁻¹ (Table II). The largest response episode measured had a total emission of 2.9×10^{11} photons episode⁻¹.

Higher rates of stimulation (10 and 20 Hz) actually resulted in decreased emis-

TABLE II

Response kinetics and quantum emission of response episodes of Pleuromamma xiphius stimulated with repetitive electrical stimuli (10 V, 5 ms duration) at frequencies ranging from 0.5 to 20 Hz

Frequency (Hz)	Latency† (ms)	Response duration (s)	Total quantum emission (photons episode ⁻¹)	Maximum response (photons episode ⁻¹)	n
0.5	36.0* ± 18.2	>17.1** (2.2)†† ± 2.3 (±0.4)	$5.2 \times 10^{10**}$ ± 1.8×10^{10}	$1.6 \times 10^{11**}$	9
1	12.2 ± 3.0	>13.8** ± 2.5	$11.0 \times 10^{10**}$ ± 4.9×10^{10}	$2.9 \times 10^{11**}$	6
2	17.1 ± 5.4	8.1 ± 1.4	7.2×10^{10} ± 1.4×10^{10}	2.5×10^{11}	11
5	21.7 ± 6.6	8.8 ± 1.8	8.0×10^{10} ± 2.1×10^{10}	2.0×10^{11}	12
10	18.8 ± 4.3	7.5 ± 1.3	5.2×10^{10} ± 1.1×10^{10}	1.0×10^{11}	10
20	33.2 ± 8.4	5.4 ± 1.3	4.8×10^{10} ± 1.3×10^{10}	1.0×10^{11}	10

* Values represent mean ± standard error of the mean.

** The reported value underestimates the actual value since some responses persisted beyond the 20 s data collection period.

† Stimulus-response latencies for different stimulus frequencies were not significantly different from one another (*t*-test, *P* > 0.05).

†† Measurements from single flashes comprising the entire response episode.

sion; average duration of the response episode was less than 6 s with a quantum emission averaging 5×10^{10} photons episode⁻¹ (Table II). Summation was so complete that individual flashes comprising the response episode were not recognizable (Fig. 5D). In fact, the resultant waveform was similar to the flash response for single pulse stimulation (Fig. 2A), although of longer duration and with greater quantum emission.

Since stimulus-response latencies were unaffected by stimulus magnitude and frequency, the values for all experiments were pooled (Fig. 6). The most common latency values occurred between 5 and 20 ms with a median latency of 16 ms (*n* = 152). This is similar to latencies of 7 to 9 ms reported for the Metridiidae (David and Conover, 1961; Clarke *et al.*, 1962). Minimum flash latencies of 4 to 18 ms have been reported for other species of the Metridiidae (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972); in the present study 6% of all specimens tested exhibited latencies less than 5 ms. The briefest flash latency measured was 2 ms, probably reflecting a direct electrical effect on the light organ (Baguet, 1975; Baguet *et al.*, 1980) rather than one mediated through sensory or central pathways, since synaptic delay alone accounts for approximately 1 ms (Katz and Miledi, 1965) in chemically transmitting crustacean neuromuscular synapses.

Observations suggested that subsequent flashes from a specimen exhibited different flash kinetics and quantum emission from those of first flashes. This was tested by subjecting 5 specimens to a second 10 V, 5 ms duration pulse approximately 4 minutes following an original stimulus pulse of the same magnitude (Table III). While statistically not significantly different (*t*-test, *P* > 0.05), all specimens exhibited a second flash response that occurred after a longer latency, had a longer rise time, and

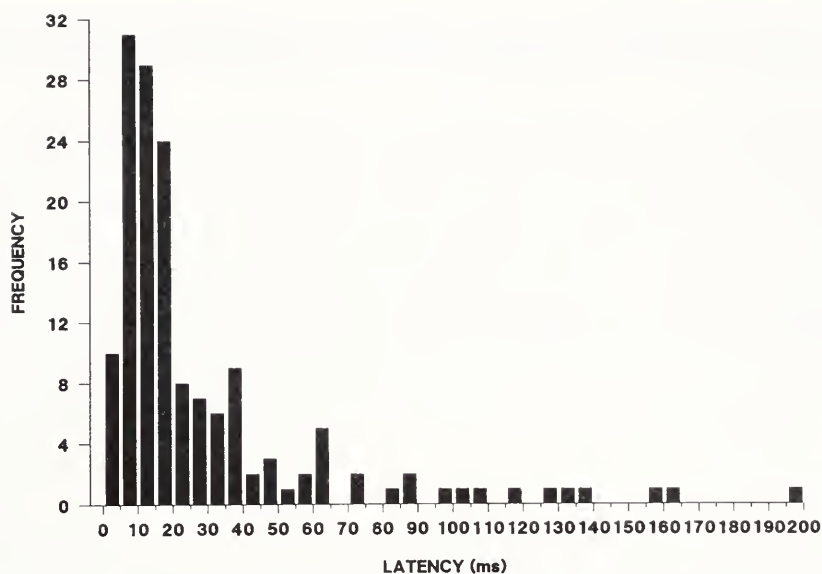


FIGURE 6. Frequency histogram of the distribution of stimulus-response latencies for 152 specimens of *Pleuromamma xiphias*. The statistical mode of the distribution was 16 ms.

was of shorter duration than the first flash. In addition there was an average 35% reduction in quantum emission for the second flash.

DISCUSSION

The most striking feature of the bioluminescence of freshly collected specimens of the copepod, *Pleuromamma xiphias*, was the variability in spectral and temporal characteristics and quantum emission of the flash response. Such variability has been

TABLE III

Comparison of response kinetics of flashes of *Pleuromamma xiphias* between initial single electrical pulse (10 V, 5 ms duration) and an identical stimulus delivered 4 min later

Latency (ms)	Total rise time (ms)	50% decay time (s)	Total flash duration (s)	Total quantum emission (photons flash ⁻¹)
A. First stimulus				
9.0*	103.1	1.4	3.0	1.6×10^{10}
± 2.4	± 24.2	± 0.6	± 0.6	± 0.7×10^{10}
B. Second stimulus**				
21.8	212.5	0.3	2.0	0.6×10^{10}
± 5.1	± 75.6	± 0.2	± 0.6	± 0.4×10^{10}

* Values represent mean ± standard error of the mean for 5 specimens.

** While mean values for flash kinetics and total quantum emission were not significantly different from those of the first flash response (*t*-test, $P > 0.05$), for each specimen the second response always had a longer latency, slower rise time, faster decay time, shorter flash duration, and diminished flash quantum emission (Sign test; Zar, 1974).

noted but not investigated in previous studies of copepod flashing (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972; Lapota and Losee, 1984).

Emission spectra

The spectral distributions of *Pleuromamma xiphius* bioluminescence were either bimodal or unimodal. Herring (1983) measured bimodal spectra in *P. borealis*, but the luminescence for other copepod genera which have been studied exhibits unimodal distributions (David and Conover, 1961; Herring, 1983; Widder *et al.*, 1983). *P. xiphius* contains 11 luminescent glands (Giesbrecht, 1895; Clarke *et al.*, 1962) of unknown variability in spectral emissions. Since variability in the recruitment of luminescent glands is well known for copepods (David and Conover, 1961; Barnes and Case, 1972), there may be a spatial origin to the variability in the emission spectra. Observation of the sites of light emission during spectral measurements is necessary to determine whether luminescence from different body regions has different spectral properties.

Flash kinetics

Luminescence by calanoid copepods is believed to occur by the expulsion of the contents of paired sacs comprising the luminescent gland through a pore and subsequent mixing outside the body (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972; Herring, 1985). The activation/expulsion process presumably involves at least three steps: (1) activation within the photocytes, (2) transport through internal channels, culminating in (3) expulsion through pores to outside the body.

In the present study the flash of *P. xiphius* was resolved into two components. The fast component of the flash appears to involve steps (1) and (2) above, with little or no expulsion from the body. Two glands are responsible for light emission: the caudal organ located on the posterior tip of the abdomen, and one or more of several thoracic light organs. The slow flash component consists of all three steps, with considerable spewing of luminescent material from the posterior light organ into the external environment. Although both components exhibit first order exponential decay, the different decay rates also suggest that the two components are separate events involving different emission mechanisms.

The wide range of values measured for the rise time of *P. xiphius* flashes (mean 170 ms, range 15 to 535 ms) reflect the variability known for copepods. Previous measurements for the Metridiidae range from 30 to 900 ms (Clarke *et al.*, 1962; Barnes and Case 1972; Lapota and Losee, 1984). The variability of these measurements may result from the presence of a rapid, neurally triggered response coupled with a mechanism with an inherently slower temporal element, such as glandular mixing and expulsion.

Flash durations for *Pleuromamma xiphius* were stimulus-dependent and extended over 2 orders of magnitude, ranging from 100 ms to greater than 20 s. Previous measurements of flash durations for *Pleuromamma* species range from 200 ms to over 6 s (Clarke *et al.*, 1962; Yevstigneyev, 1985), and 100 ms to 11 s for other copepods (Barnes and Case, 1972; Lapota and Losee, 1984). In this study the maximum period over which luminescence could be expressed was not investigated; however, the copepod *Gaussia* is able to respond to single electrical pulses delivered at 0.1 Hz for more than 3 min (Barnes and Case, 1972).

Quantum emission

Considering the abundance of luminescent plankton, there are few measurements available of the quantum emission of individual flashes, in part due to the difficulty

TABLE IV

Total quantum emission of single flashes from planktonic organisms

Organism	Mode of stimulation	Mean total quantum emission (photons flash ⁻¹)	Reference
Protozoa			
Colonial radiolaria	Mechanical	1×10^9	Latz <i>et al.</i> , 1987
Dinophyta			
<i>Noctiluca miliaris</i>	Electrical	2×10^9	Eckert, 1967
<i>Ceratium horridum</i>	Vacuum*	4×10^7	Lapota and Losee, 1984
<i>Ceratium breve</i>	Vacuum	1×10^8	Lapota and Losee, 1984
<i>Pyrocystis noctiluca</i>	Mechanical	7×10^9	Latz and Case, unpub.
<i>Pyrocystis fusiformis</i>	Mechanical	2×10^{10} first flash 0.1×10^{10} subsequent flash	Latz and Case, unpub.
<i>Gonyaulax polyedra</i>	Spontaneous	1×10^7	Latz and Case, unpub.
Crustacea			
Euphausiacea			
<i>Euphausia eximia</i> calyptopis I	Vacuum	1×10^{10}	Lapota and Losee, 1984
<i>Nyctiphanes simplex</i> furcilia I	Vacuum	6×10^{10}	Lapota and Losee, 1984
<i>N. simplex</i> furcilia III	Vacuum	1×10^{11}	Lapota and Losee, 1984
Copepoda			
<i>Pleuromamma xiphias</i>	Single electrical Repetitive elect.	1×10^{10} 9×10^{10}	Present study
<i>Corycaeus latus</i>	Vacuum	1×10^8	Lapota and Losee, 1984
<i>Centropages furcatus</i>	Vacuum	8×10^7	Lapota and Losee, 1984
<i>Corycaeus speciosus</i>	Vacuum	5×10^7	Lapota and Losee, 1984
<i>Paracalanus indicus</i>	Vacuum	3×10^7	Lapota and Losee, 1984
Ostracoda			
<i>Conchoecia secernenda</i>	Electrical	3×10^{10}	Latz, Frank, and Case, unpubl.

* Flashes were induced by removing water from chamber, stranding organisms on filter paper.

in making these measurements. Electrical stimulation allows for precise control of stimulus parameters such as pulse strength, duration, and frequency, and the results of the present study have demonstrated that changes in these parameters greatly influence the resulting quantum emission of the luminescent responses. Quiescent specimens of *P. xiphias* were extremely sensitive to handling; it was not uncommon for some bioluminescence to be triggered during handling. Therefore, the present measurements of flash output are conservative estimates of luminescence capacity.

Light emission by planktonic organisms for which data are available (Table IV) ranges from 1×10^7 photons flash⁻¹ for the dinoflagellate *Gonyaulax* (Latz and Case, unpub.) to approximately 1×10^{11} photons flash⁻¹ for larval euphausiids (Lapota and Losee, 1984). The quantum emission of luminescence by *P. xiphias* is situated at the upper portion of this range, and is 1.5–2.5 orders of magnitude greater than that reported for smaller copepods by Lapota and Losee (1984), although these differences may also reflect differing excitational and recording methods as well as species differences.

The present data on the total quantum emission of *Pleuromamma xiphias* luminescence reflect the variability in responses previously observed in the Metridiidae (David and Conover, 1961; Clarke *et al.*, 1962; Barnes and Case, 1972). The maximum total quantum emission measured following electrical stimulation was equivalent to the bioluminescence potential, or total luminescent capacity of *P. xiphias*, which, based on measurements of total mechanically stimulated bioluminescence, is estimated to be approximately 1×10^{11} photons (Latz and Case, unpub.). Therefore

a single flash represented approximately 4–15% of total possible light emission. Thus, *P. xiphius*, as well as other copepods, is capable of emitting numerous flashes before response failure occurs (Clarke *et al.*, 1962; Barnes and Case, 1972; present study). In the present study, electrical stimulation at 2 Hz effectively evoked the total luminescent capacity, while other stimulus frequencies generated flash episodes which utilized 50% or more of total luminescent capacity.

Implications of flash variability

Previous studies of copepod luminescence have noted the “irregular” shapes of the flash responses (Clarke *et al.*, 1962; Barnes and Case, 1972). Generally, response episodes to repetitive stimulation exhibited more complex and variable waveforms than flashes induced by single stimuli. This trend was also true for *P. xiphius*. For this organism, stimulus frequency was a predictable source of variability of the response waveform. However, the general pattern was the higher the stimulus frequency, the simpler the flash waveform, due in part to different degrees of temporal summation of individual flashes comprising the response episode. There was no standard response waveform for all stimulus protocols.

Such a large capacity for luminescence, plus the variability in flash waveform and quantum emission, suggest that copepods are not limited to a single type of flash but instead may exhibit a versatility of luminescent behaviors. Behavioral versatility in zooplankton-secreted luminescence is in fact known for coastal ostracods, for which three types of behaviorally significant luminescent displays have been described (Morin and Bermingham, 1980; Morin, 1986). These displays involve not only control of the interval between flashes but also of flash duration, the latter through mechanisms that may involve differences in the chemical composition of the luminescent secretion. In some species individual ostracods produce dozens of flashes during the signaling sequence. Evidence for behavioral versatility of luminescent displays also exists for squid (Young *et al.*, 1982), fish (McFall-Ngai and Dunlap, 1983), and for counter-illuminating organisms (reviewed by Young, 1983).

There is evidence for multiple modes of copepod luminescence. *Metridia* generates two types of flashes during feeding experiments with euphausiids as predators (David and Conover, 1961). Single bright flashes may be associated with an escape response, while multiple flashes over a 30-s interval may be associated with successful predation.

In terms of the present measurements of the physical characteristics of the flash of *Pleuromamma xiphius*, variability in the emission spectra, flash kinetics and flash quantum emission most likely result from a combination of several factors: spatial origin of emission from the body, temporal summation and complex neural processes (as determined by stimulus strength and frequency), previous excitation history, collection and handling artifacts, and general physiological state of the organism. Until additional experiments can correlate flash patterns with specific behaviors, the variability in bioluminescence of *P. xiphius* only suggests the physiological mechanisms responsible for possible differences in luminescent displays.

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QUANTITATIVE ESTIMATION OF MOVEMENT OF AN AMINO ACID FROM HOST TO *CHLORELLA* SYMBIONTS IN GREEN HYDRA

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ABSTRACT

Washing symbiotic *Chlorella* algae freshly isolated from green hydra with 0.05% sodium dodecyl sulphate was shown to remove virtually all contaminating host material, previously a severe constraint in quantifying movement of metabolites from host to symbionts. When brine shrimp labelled with ^3H -leucine were fed to hydra in symbiosis either with the native strain of *Chlorella* (E/E hydra) or two strains cultured from *Paramecium bursaria* (E/3N and E/NC hydra), it was found that after 24 h 3–4% of the total radioactivity retained by the symbiosis was present in the algae. Analysis of the free amino acid pool of symbiotic algae from E/E hydra showed that over 70% of the radioactivity was associated with leucine, and significant amounts of radioactivity were retained by these algae for at least five days following a single feeding with radioactive brine shrimp. In both E/E and E/NC hydra, the amount of radioactivity per unit protein was considerably less in the symbionts than in the host, suggesting that access to host amino acid pools were limited. These results are discussed in terms of the possible role and regulatory significance of amino acids as a nitrogen source to symbiotic *Chlorella*, and of the cost to the host in maintaining the symbiosis.

INTRODUCTION

While it is well known that *Chlorella* algae symbiotic with green hydra release photosynthetically fixed carbon that is used by their hosts (Cernichiari *et al.*, 1969; Mews, 1980; Mews and Smith, 1982), it has become apparent only recently that movement of metabolites in the opposite direction may also be important in maintaining the symbiosis. In hydra grown in darkness, host supply of metabolites, which from indirect evidence may include glucose (McAuley, 1986a), is necessary to maintain a reduced population of symbionts. In light, cell division of the algae appears to be dependent upon a 'factor' present in host food, possibly one or more amino acids (McAuley, 1985, 1986b).

Amino acids may also have an important role in nitrogen supply to the symbionts. Although it was long believed that algae symbiotic with green hydra used ammonium produced by host catabolic processes, as in symbioses between marine invertebrates and zooxanthellae (Muscatine, 1980; Wilkerson and Muscatine, 1984; Summons *et al.*, 1986; Anderson and Burris, 1987), recent research has shown that high levels of host glutamine synthetase and low perialgal vacuolar pH may prevent uptake of ammonium by algae in green hydra (Rees, 1986). Since the symbiotic algae also lack nitrate reductase and nitrite reductase (D. McKinney and H. Lenhoff, University of California, Irvine, pers. comm.), it has been suggested that host supply of amino acids

may form the primary source of nitrogen for algae symbiotic with green hydra (McAuley, 1986b, 1987; Rees, 1986).

Direct evidence for movement of metabolites from host to symbiotic algae has been difficult to obtain. Logically, it could be studied by supplying food containing radioactively labelled metabolites, then isolating algae from the symbiosis and assessing uptake of radioactivity. Although this approach has been used (Cook, 1972; Thorington and Margulis, 1981), the interpretation of the results is now in doubt because it was subsequently realized that algae isolated by centrifugation of homogenates of green hydra are heavily contaminated with host material, despite washing the algal pellet in several changes of distilled water or culture medium (Cook, 1983; Douglas and Smith, 1983; McAuley, 1986c).

Recently, it was demonstrated that complete removal of contaminating host material could be achieved by washing the algal pellet with a dilute solution (0.05%, w:v) of the surfactant sodium dodecyl sulphate (SDS) (McAuley, 1986c). Meints and Pardy (1980) earlier used SDS to isolate algae from green hydra, but at a higher concentration which was subsequently found to affect the viability of the algae. However, neither the viability, nor the photosynthetic capacity, nor the ability to sequester and retain amino acids were affected after washing with 0.05% SDS (McAuley, 1986c; Douglas, in press). In this paper the SDS-washing technique was used to separate algae from host material in homogenates of hydra which had been supplied with food labelled with ^3H -leucine. Evidence for the transfer of leucine from host food to the symbiotic algae is described and discussed.

MATERIALS AND METHODS

Maintenance of organisms

Green and aposymbiotic hydra of the European strain of *Hydra viridissima* PAL-LAS were cultured in M solution (Muscatine and Lenhoff, 1965) at 18°C in continuous light ($60 \mu\text{Einsteins m}^{-2} \text{s}^{-1}$). Cultures were fed each Monday, Wednesday, and Friday with freshly hatched nauplii of the brine shrimp *Artemia salina* (Lenhoff and Brown, 1970). Green hydra were symbiotic with either the native strain of *Chlorella* algae (E/E hydra) or were derived from aposymbionts artificially reinfected with NC64A (E/NC hydra) or 3N813A (E/3N hydra) strains of *Chlorella* originally isolated from *Paramecium bursaria* (Muscatine *et al.*, 1967; Weis, 1978). All experimental hydra had been fed 72 h prior to use and each bore a single bud. Cultured 3N813A algae were grown as previously described (McAuley, 1986a).

Radioactive labelling of brine shrimp

Freshly hatched nauplii of *Artemia salina* were incubated at a density of 30 nauplii ml^{-1} in artificial seawater (Tropic Marin: Dr. Beiner Aquarientechnik, Warthenberg, W. Germany) containing $0.5 \mu\text{Ci ml}^{-1}$ L-[4,5- ^3H] leucine (Amersham International plc, England). After 24 h in darkness at 25°C (normal hatching conditions), *Artemia* were washed with M solution and added to dishes containing hydra (two shrimp per hydra). Determination of radioactivity in homogenates of *Artemia* showed that on average each contained 7260.7 ± 390.3 dpm (mean $\pm$ S.E. of five separate determinations). Almost 80% of the label was associated with TCA-ethanol insoluble material, suggesting that most of the leucine had been incorporated into protein.

Separation of radioactivity in algal and animal fractions of hydra

Twenty-four hours after being fed with ^3H -leucine labelled *Artemia*, hydra were washed in M solution, homogenized, and the volume made up to 1.5 ml. Three 100- μl aliquots of homogenate were immediately counted (total radioactivity), a further three were taken to determine total protein content, and numbers of algae were determined by counting a diluted aliquot using a hemocytometer. The algae in the rest of the homogenate were washed with 0.05% (w:v) SDS and distilled water (McAuley, 1986c), slightly modifying the technique to take account of the small volumes used. The washed algal pellet was finally resuspended in 0.45 ml distilled water. Three 100- μl aliquots were counted for radioactivity and a further 100 μl was diluted and numbers of algae determined so that dpm per SDS-washed algal cell could be calculated. Radioactivity was measured by making aliquots up to 1 ml with distilled water in a plastic scintillation vial, adding 8 ml scintillation fluid (1000 ml toluene, 500 ml Triton X-100, 6 g 2-5-diphenyloxazole), and counting using a Beckman LS1801 scintillation counter. Disintegrations per minute were calculated using the H number method.

Protein content of homogenates and SDS washed algae

Samples were freeze-thawed, extracted with an equal volume of 0.4 M NaOH for 1 h, and protein content determined as previously described (McAuley, 1986c).

Thin layer chromatography

Ethanolic extracts of SDS-washed algae were spotted on a TLC plate together with a small volume containing unlabelled authentic amino acids. Chromatograms were run as previously described (McAuley, 1987). Amino acids were identified by spraying with ninhydrin; spots were scraped off and suspended in scintillation fluid (1000 ml toluene, 5 g 2-5-diphenyloxazole, 0.3 g (1,4-bis[5-phenyl-2-oxazolyl]-benzene; 2,2'-p-phenylene-bis[5-phenyloxazole], and radioactivity counted.

RESULTS

Effectiveness of SDS-washing in removal of animal contamination

To test if SDS-washing removed significantly more animal contamination from the algal pellet than simply washing with distilled water, E/E hydra were fed ^3H -leucine labelled *Artemia*, homogenized after 24 h, and the algae in the homogenate washed either with distilled water (controls) or 0.05% SDS. The algal pellets washed with distilled water contained almost nine times the radioactivity of those washed with SDS, while comparison of the amount of label per algal cell (as recovery of algae is lower after SDS-washing) showed that controls appeared to contain six times as much radioactivity as algae washed with SDS (Table I). To test if SDS washing removed only radioactivity associated with contaminating host material, and not radioactivity already sequestered by the algae, cultured 3N813A algae (10^7 cells per ml in 20 mM phosphate buffer, pH 6.3) were incubated in 0.1 mM ^3H -leucine (5 mCi per mmol) for 1 h, diluted with ice cold buffer to stop uptake, and then washed either with SDS and distilled water, or with distilled water only. Aliquots were filtered onto glass fiber disks and radioactivity was determined as previously described (McAuley, 1986a). Radioactivity per cell in algae washed with either SDS or distilled water was compared to that in algae filtered immediately at the end of the incubation period (controls). Paired *t*-tests of four replicate experiments showed no significant differ-

TABLE I

Comparison of apparent uptake of ^3H -label by algae isolated from hydra fed with ^3H -leucine labelled shrimp by conventional or SDS-washing techniques

	dpm/100 μl algal pellet	no algae $\times 10^5$ / 100 μl pellet	dpm/algal cell
<i>Wash</i>			
Distilled water	9537.6 $\pm$ 1390.1	7.241 $\pm$ 0.895	0.01318 $\pm$ 0.00142
SDS	830.3 $\pm$ 68.6	3.716 $\pm$ 0.451	0.00223 $\pm$ 0.00032

Eighty hydra were homogenized 24 h after being fed with ^3H -leucine labelled *Artemia* and aliquots of the homogenate were washed with distilled water or SDS and numbers of algae and radioactivity determined as described in Materials and Methods. Figures are means $\pm$ S.E. of four independent experiments.

ence ($P > 0.10$) in radioactivity retained by controls (0.01050 ± 0.00099 dpm per cell) compared to radioactivity retained after washing with either SDS (0.00993 ± 0.00096 dpm per cell) or distilled water (0.01014 ± 0.001172 dpm per cell). Therefore, it was concluded that loss of radioactivity from algae during SDS washing was insignificant.

Further experiments determined whether all label associated with contaminating host material could be removed from the algal pellet by SDS-washing. Aposymbiotic hydra were homogenized 24 h after having been fed with ^3H -leucine labelled *Artemia*, and appropriate numbers of unlabelled cultured 3N813A algae were suspended in the homogenate and washed either with distilled water or with SDS. Counts showed that over 10% of radioactivity added to the algae remained after washing with distilled water, but less than 0.2% remained after washing with SDS (Table II). The results in Tables I and II suggest that if algae are washed only with distilled water, 80% of the radioactivity apparently associated with them is due to animal contamination. This is almost entirely removed by washing with SDS. A similarly high level of contamination was measured in zooxanthellae isolated from tentacles of specimens of the sea anemone *Aiptasia pulchella* which had been fed *Artemia* labelled with ^{35}S -methionine (Steen, 1986a).

TABLE II

Effect of SDS washing on removal of contaminating aposymbiotic hydra homogenate from unlabelled cultured 3N813A cells

	Experiment 1		Experiment 2	
	dpm	%	dpm	%
Label added/100 μl algae recovered	19821.9 $\pm$ 125.8	100.00	22562.2 $\pm$ 235.2	100.00
Label/100 μl algae washed with distilled water	2127.8 $\pm$ 40.7	10.74	3019.6 $\pm$ 36.8	13.38
Label/100 μl algae washed with 0.05% SDS	8.6 $\pm$ 0.5	0.04	33.7 $\pm$ 2.6	0.15

Appropriate numbers of unlabelled cultured 3N813A cells were suspended in homogenate of aposymbiotic hydra which had been fed with ^3H -leucine labelled *Artemia* 24 h previously. Radioactivity was determined before and after washing algal cells with distilled water or 0.05% SDS. Figures are means $\pm$ S.E. of four replicate aliquots.

TABLE III

Partition of ³H-label in green hydra

	Total dpm/100 μ l homogenate	dpm/algal cell	dpm/total algae in 100 μ l	% label in algae
<i>Symbiosis</i>				
E/E	34803.3 $\pm$ 3575.9	0.003493 $\pm$ 0.00030	1191.4 $\pm$ 141.5	3.4
E/3N	30574.3 $\pm$ 2820.7	0.005592 $\pm$ 0.000564	1304.2 $\pm$ 178.7	4.3
E/NC	24263.2 $\pm$ 2694.9	0.002220 $\pm$ 0.000193	694.0 $\pm$ 53.7	2.9

Forty hydra were homogenized in 1.5 ml 24 h after having been fed ³H-leucine labelled *Artemia*, and label in animal and algal fractions determined as described in Materials and Methods. Figures are means $\pm$ S.E. of four independent experiments.

Partition of ³H-label supplied to green hydra by holozoic feeding

To measure movement of amino acid from host food to symbiotic algae, green hydra were fractionated (see procedure in Materials and Methods) 24 h after being fed with ³H-leucine labelled *Artemia*. Transfer of ³H-label to symbiotic algae occurred both in the normal (E/E) symbiosis and in artificial (E/3N and E/NC) symbioses, amounting to 3–4% of the total radioactivity retained by the hydra (Table III). In one experiment, amino acids were extracted in 80% ethanol from algae isolated from E/E hydra which had been fed ³H-leucine labelled shrimp 24 h previously. Extract was co-chromatographed with authentic markers, and ninhydrin positive spots were scraped off and radioactivity determined. About 10% of identified ³H-label in the ethanol-soluble pool was arginine, about 7% alanine, 3–5% each of aspartate, glutamine, and glutamate, and over 70% leucine. These results suggest that symbiotic algae were able to take up amino acids supplied to green hydra via holozoic feeding.

Further comparisons were made on the basis of specific activities per unit animal and algal protein in E/E and E/NC hydra. The total amount of protein in samples was determined directly, while the amount in algal cells was calculated from separate determinations of algal cell protein content and estimation of numbers of algae per sample. Calculations showed that in both symbioses, algal cells contained less radioactivity per unit protein than hydra tissue (Table IV). The amount of radioactivity per unit protein in E/E algae was about 24%, and in E/NC algae 18%, that per unit

TABLE IV

Partition of ³H-label in green hydra according to protein content of animal and algal fractions

	Protein/algal cell (pg)	Protein in algae/ 100 μ l homogenate (μ g)	dpm/ μ g algal protein	Protein in animal/ 100 μ l homogenate	dpm/ μ g animal protein
<i>Symbiosis</i>					
E/E	13.08 $\pm$ 0.14	4.44 $\pm$ 0.33	268.6 $\pm$ 23.0	31.61 $\pm$ 2.66	1109.8 $\pm$ 202.7
E/NC	10.85 $\pm$ 0.43	3.44 $\pm$ 0.33	204.6 $\pm$ 17.8	21.99 $\pm$ 2.49	1145.7 $\pm$ 229.5

Protein content of algal cells measured in three (E/NC) or four (E/E) separate determinations in 2–4 replicate samples containing 5×10^6 SDS-washed algae. Protein content of animal and algal fractions determined from measurements of total protein/100 μ l homogenates (from experiments described in Table III), number of algae/100 μ l and protein content of algal cells. Figures are means $\pm$ S.E. of four independent experiments.

protein of hydra tissue. This suggested that availability to the algae of amino acid derived from holozoic feeding was restricted, either because of some host mechanism directly controlling supply, or because a large proportion was immediately sequestered by host cells upon entry.

Retention of ^3H -label by symbiotic algae

The amount of radioactivity retained by symbiotic algae was measured for five days subsequent to feeding green hydra with ^3H -leucine labelled *Artemia*. At 24-h intervals, 40 hydra were homogenized, total dpm in aliquots of homogenate and per SDS-washed alga were determined, and the percentage of the total dpm found in the algae was calculated as described in Materials and Methods. Net uptake by the algae of radioactively labelled amino acid was found to continue up to 48 h after feeding (Fig. 1), but thereafter dpm per algal cell slowly declined. In contrast, the percentage of the total label retained by the symbiosis which was found in the algal population continued to increase over the period of measurement. This difference may be due to either of two reasons. First, uptake of amino acid by the algae could have been diluted by algal cell division; although algal mitosis reaches a maximum about 12 h after host feeding, it continues, although at a declining rate, for several days thereafter (McAuley, 1982). Second, increase in the proportion of label retained by algae despite a fall in dpm per algal cell may be a result of differences in the ability of algal cells and animal tissue to retain radioactively labelled amino acid. Possibly, the rate of protein catabolism was faster in host cells than in symbiotic algae during host starvation.

DISCUSSION

Contamination of the algal pellet with host material has previously been a severe constraint on determining how radioactive metabolites are partitioned between the

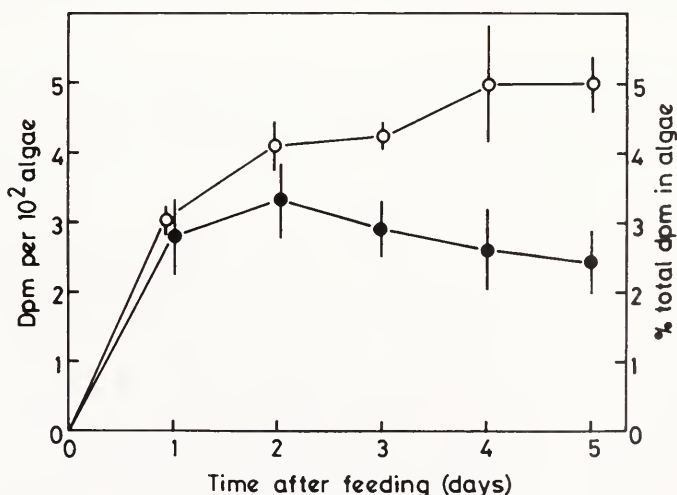


FIGURE 1. Retention of ^3H -label by symbiotic algae in E/E hydra fed ^3H -leucine labelled *Artemia*. Hydra were fed radioactive brine *Artemia* as described in Materials and Methods. At daily intervals 40 hydra were homogenized and dpm per algal cell (● — ●) and % total ^3H -label retained by the symbiosis found in algal cells (○ — ○) were calculated. Each point is the mean $\pm$ S.E. of three independent experiments.

host and its symbionts in algal/invertebrate symbioses. Although conventional washing by repeated centrifugation and resuspension in fresh medium will remove up to 90% of contaminating host material from algae isolated from green hydra (Cook, 1972; Douglas and Smith, 1983), the remaining 10% may contain several times the radioactivity actually sequestered by the algae. Contamination represents at least the same amount of protein as contained by algal cells (Douglas and Smith, 1983; McAuley, 1986a), and results described here showed that host material contained three to four times as much radioactivity per unit protein as did the algae. Therefore, unless contamination is accurately quantified or completely removed, uptake of radioactivity by symbiotic algae *in vivo* is liable to be severely overestimated.

In our experiments, SDS-washing was shown to remove from the algal pellet all but a fraction of 1% of radioactive label associated with host contamination. This enabled accurate measurement of the partition of label between host and symbiotic algae in hydra that had been fed with ^3H -leucine labelled *Artemia salina* nauplii. In both the natural symbiosis (E/E) and in artificial symbioses with *Paramecium* algae (E/NC, E/3N), radioactivity moved into the algae after hydra were fed with ^3H -leucine labelled *Artemia*, and algae in E/E hydra were found to retain significant amounts of radioactivity for at least five days after feeding.

Although the algae constitute about 15% of the total protein content of the symbiosis, only 3–4% of radioactivity retained by green hydra was found in the algae after 24 h, rising to just over 5% after 120 h. This suggests that access by the algae to amino acids derived from host digestion of food may be restricted. Since amino acids must pass through the host-derived vacuolar membrane which surrounds each alga, it is possible that the host cell can directly control the rate of amino acid supply (McAuley, 1986b). Carroll and Blanquet (1984) found that a low molecular weight factor isolated from host tissue inhibited uptake of alanine by zooxanthellae symbiotic with the jellyfish *Cassiopea xamachana*. Alternatively, differences in specific labelling of algal and animal protein may be due to competition between uptake by the algae and host utilization of amino acid for protein synthesis. That algae contain less label than expected also may be due to recycling to the host of a proportion of sequestered amino acid.

Demonstration of 'reverse translocation' suggests that maintenance of the green hydra symbiosis depends on flow of metabolites in both directions. Not only is maltose exported from the algae and used by the host (Mews, 1980; Mews and Smith, 1982), but amino acids and possibly other metabolites (McAuley, 1986a) derived from host feeding are transported into the perialgal vacuole and used by the symbiotic algae. The significance of reverse translocation is two-fold.

First, as suggested by Douglas and Smith (1983), supply of metabolites to the algae may impose a net 'cost' upon the host in certain circumstances. When hydra are grown in continuous darkness, where maltose release is reduced and the algae must depend upon host-derived metabolites for growth, those with a population of algae grow more slowly than aposymbionts artificially rid of their algae (Douglas and Smith, 1983). A similar net cost has been found in the sea anemone *Aiptasia pulchella*, whose symbiotic zooxanthellae sequester radioactivity when hosts are given ^{35}S -methionine labelled food (Steen 1986a). Symbiotic anemones starved in darkness showed a significant decline in the adenylate ratio of ATP: (ATP + ADP) compared to aposymbionts, but in light the adenylate ratio declined at the same rate in starved symbiotic and starved aposymbiotic anemones (Steen, 1986b).

Second, there is now persuasive evidence that uptake of ammonium by algae in symbiosis with green hydra is prevented by low perialgal vacuolar pH and high levels of host glutamine synthetase (Rees, 1986). Furthermore, measurements of amino

acid pool size and of uptake and metabolism of amino acids indicate that symbiotic algae may be nitrogen-limited (McAuley, 1986b, 1987). Experiments described here, demonstrating movement of ^3H -leucine from host food to symbiotic algae, support the suggestion that in green hydra amino acids may form the principal supply of nitrogen in symbiosis (McAuley, 1986b, 1987; Rees, 1986). This differs from symbioses between marine invertebrates and zooxanthellae, in which nitrogen is supplied to the symbionts as ammonium (Muscatine, 1980; Wilkerson and Muscatine, 1984; Summons *et al.*, 1986; Anderson and Burris, 1987). These experiments also suggested that *in vivo* uptake of ^3H -leucine by symbiotic algae may be restricted. Host control of supply of nitrogen, or of one or more specific amino acids, may be the mechanism whereby cell growth of symbiotic algae is reduced, and cell division is inhibited except at host cell division (McAuley, 1981, 1985, 1986d).

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NEURONAL CONTROL OF CILIARY LOCOMOTION IN A GASTROPOD VELIGER (*CALLIOSTOMA*)

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ABSTRACT

Intracellular recordings from pre-oral ciliated cells of competent *Calliostoma ligatum* veligers were used to demonstrate the mechanisms of neuronal control of ciliary locomotion. During normal ciliary beating at 5–7 Hz, the membrane potential shows no oscillations or spiking activity. It remains at a resting potential of about –60 mV. Depolarization from resting potential is due to excitatory input from the CNS and, depending upon the kind of input, veligers appear to show two types of locomotory behavior. In one type, normal ciliary beating is periodically interrupted by rapid, velum-wide ciliary arrests. These arrests are caused by a propagated, Ca^{++} -dependent action potential in the pre-oral ciliated cells. The second type is characterized by either a velum-wide or local slowing of normal ciliary beating, and appears to result from a slow depolarization of the ciliated cell membrane. Pre-oral ciliated cells are electrically coupled to each other. This property may ensure the synchrony of velum-wide ciliary arrests or differential velar slowing of ciliary beating. These findings demonstrate some of the mechanisms of the fine control veligers possess over their locomotory and feeding behavior.

INTRODUCTION

Many metazoans that use cilia to move or to produce feeding currents can control the frequency of ciliary beating. This control is often revealed as intermittent, rapid arrests of ciliary beating. Ciliary arrests have been correlated with chemically mediated, rapid, postsynaptic depolarizations of the ciliated cell membrane (Mackie *et al.*, 1974; Murakami and Takahashi, 1975; Mackie *et al.*, 1976; Moss and Tamm, 1986; Arkett, 1987). These depolarizations are rapidly conducted through gap junctions, which connect the ciliated cells (Gilula and Satir, 1971; Mackie *et al.*, 1974). Additionally, some animals appear to have a finer degree of control of ciliary beating. For example, the ciliary beating frequency of lateral cilia of *Mytilus* gill is variable and dependent upon dopaminergic and serotonergic CNS input (Paparo and Aiello, 1970; Paparo *et al.*, 1975; Aiello *et al.*, 1986). Larvae of many marine invertebrates are also capable of modulating the beat frequency of their locomotory cilia (see review by Chia *et al.*, 1984), yet the mechanisms by which gradual changes in ciliary beat frequency are generated are not well understood.

Veliger larvae use the pre-oral (locomotory) cilia of the velum to move through the water column. Upward swimming is periodically interrupted, either spontaneously or upon contact with “foreign” objects, by sudden ciliary arrests whereupon the veliger rapidly sinks (Garstang, 1929). These ciliary arrests have long been assumed to be under neuronal control because neuronal processes, emerging from the cerebral ganglion, ramify across the velum to the pre-oral ciliated cells (Carter, 1926; Carter,

1928; Werner, 1955; Fretter, 1967) and form chemical synapses with them (Mackie *et al.* 1976). Electrical activity recorded extracellularly and intracellularly from pre-oral ciliated cells shows no spiking activity during normal ciliary beating, but large depolarizations occur one-for-one with velum-wide ciliary arrests (Mackie *et al.*, 1976). These arrests are absent when the velum is isolated from the rest of the veliger. Pre-oral cell action potentials appear to be Ca^{++} -dependent since elevated Ca^{++} causes longer duration ciliary arrests and addition of Co^{++} blocks arrests (Korobtsov and Sakharov, 1971). Serotonin has a cilio-excitatory effect (Koshtoyants *et al.*, 1961) and an unknown "inhibitory substance," extracted from nudibranch veligers, causes ciliary arrest (Buznikov and Manukhin, 1962).

Some veligers are also capable of finer control of ciliary beating. This control is usually expressed as a gradual decrease in the frequency of the beating cilia. Veligers are thus able to slowly descend with cilia beating at "a reduced level" (Cragg, 1980). Furthermore, there is evidence that veligers are capable of integrating sensory information to alter locomotory and feeding behavior since the beating "vigor" and "rate of [food] collection" by pre-oral cilia varies with the degree of satiation (Fretter and Montgomery, 1968). During this study, we developed a new preparation, exploiting the large, pre-oral ciliated cells of a gastropod veliger larva, to examine the mechanisms by which the larval nervous system modulates ciliary beating and hence controls locomotion.

We demonstrate that excitatory input from the CNS modulates the inherent beating frequency of pre-oral cilia of *Calliostoma ligatum* (Gould, 1849) veligers. We also show that the ciliated cells are electrically coupled to each other, a property which may ensure the velum-wide synchronization of rapid ciliary arrest. These features endow the veliger with two distinct types of locomotory behavior.

MATERIALS AND METHODS

Larvae were raised in the laboratory. Adult *Calliostoma ligatum*, collected from San Juan Island, Washington, were spawned in separate bowls, containing 20°C, coarse-filtered seawater. Animals were placed foot up and usually spawned between 30 min to 1 hour later. Gelatinous egg strands were dissociated by repeatedly drawing the egg mass into a Pasteur pipette. Eggs were then transferred to additional bowls at a concentration of several hundred eggs per bowl. Approximately 2 ml of sperm from several different males were diluted in 500 ml of filtered seawater. Eggs were fertilized by adding about 0.5 ml of diluted sperm to each bowl. Developing veligers were kept on a seawater table at 10–12°C. Seawater in the bowls was changed twice daily and unfertilized eggs and moribund veligers were removed. Veligers developed rapidly within their egg capsules and usually hatched and began swimming around the bowl after about 5 days. We found that the electrical properties of pre-oral ciliated cells and ciliary activity of veligers as young as 72 hours post-fertilization were nearly identical to that of older veligers, but younger veligers did not retract into their shell as readily. For this reason, we often used these younger veligers for intracellular recordings after excapsulating them with sharpened tungsten needles.

Hatched or excapsulated larvae were held in position and manipulated for recording by attaching a small bore suction (polyethylene tubing) electrode to the shell or foot, or by placing the shell into a small depression in the Sylgard- (Dow Corning) lined recording dish. The frequency of ciliary beating was monitored by holding a larva in the beam of a low power laser (Spectra Physics Model 155; 0.95 mW, 632.8 nm). The beam was aimed at a photodiode and the beating cilia on one velar lobe alternately bisected the beam (Fig. 1D). Voltage changes across the photodiode were

amplified by an AC-coupled preamplifier (Grass Model P-15) with the low pass filter set for 1 s (the longest τ possible). Stroboscopic measurements of changes in ciliary beat frequency were made with a Chadwick-Helmuth Strobex. Conventional intracellular recording techniques were used to record from pre-oral ciliated cells (3 M KCl-filled glass electrodes; 20–30 M Ω). For dye injection, electrodes were tip-filled with 5% Lucifer Yellow CH (Sigma) in distilled water and back-filled with 1 M LiCl₂ (90–100 M Ω). Dye was injected with pulsed hyperpolarizing current (0.5–1.0 nA) for up to ten minutes. Lucifer Yellow-filled velar cells were viewed live with a fluorescence microscope and photographed. All recordings were made in 15–18°C seawater unless otherwise stated. Artificial seawater was used for experiments requiring altered ionic content. Normal artificial seawater was composed of 430 mM NaCl, 10 mM CaCl₂, 10 mM KCl, 30 mM MgCl₂, 20 mM MgSO₄, 10 mM Tris HCl pH = 7.8.

Larvae were processed for electron microscopy by first anesthetizing them in a 1:1 mixture of seawater and isotonic (0.33 M) MgCl₂ and then fixing in a solution containing 2.0% TEM grade formaldehyde, 1.5% glutaraldehyde, 0.2 M sodium phosphate (monobasic), 0.2 M sodium phosphate (dibasic) buffer, pH = 7.2 on ice. Larvae were rinsed, post-fixed in 2% osmium tetroxide in the same buffer, dehydrated through graded alcohols and propylene oxide, and embedded in EPON 812. Sections were stained with uranyl acetate and lead citrate.

RESULTS

Ciliary beating and arrest behavior

The pre-oral cilia of *C. ligatum* larvae beat in laeoplectic metachronal waves (Fig. 1A, B) with a frequency of 5–7 Hz (Fig. 1D). This ciliary beating propels larvae upward with the velum and foot leading and the shell trailing. Stoppages of the pre-oral cilia (ciliary arrest) occur either spontaneously, or when larvae contact the air-water interface or other obstacles. Ciliary arrest is characterized by a synchronized stoppage of all pre-oral cilia and varying degrees of contraction of the velar lobes. Cilia are held in a cone-shaped tuft over the disc of the velum (Fig. 1c) during arrest, and thus reside at the beginning of the effective stroke. After a few seconds, the pre-oral cilia begin to beat and the metachronal wave is re-established. Although the arrest of all cilia is synchronized, the resumption of ciliary beating is not always uniform around the velum. Clearly the cilia of some portions of the velum start beating before others. Strong or repetitive stimuli may also cause a withdrawal of the velum and foot into the shell. Recovery from this contracted position usually takes longer than recovery from a simple ciliary arrest.

Ciliary arrests, exhibited by encapsulated or free-swimming larvae, are often rhythmical. A sampling of ten different larvae, all of which were about 96 hours old and still in egg capsules, showed 13.9 to 22.1 ciliary arrests per minute. The interval between arrests is usually very regular. As an example, one larva showing an arrest frequency of 17.6 arrests/min had a mean ($\pm$ SE, $n = 31$) period of 3.46 (0.07) seconds. Free-swimming veligers also showed rhythmical arrests, but the rhythmicity was often interrupted by external factors such as debris, air-water interface, or other veligers. In addition to these coordinated velum-wide arrests, we have observed a general slowing of ciliary beating. The slowing in the sinking rate or upward swimming that we have observed in free-swimming veligers appears to be due to this general slowing of ciliary beating.

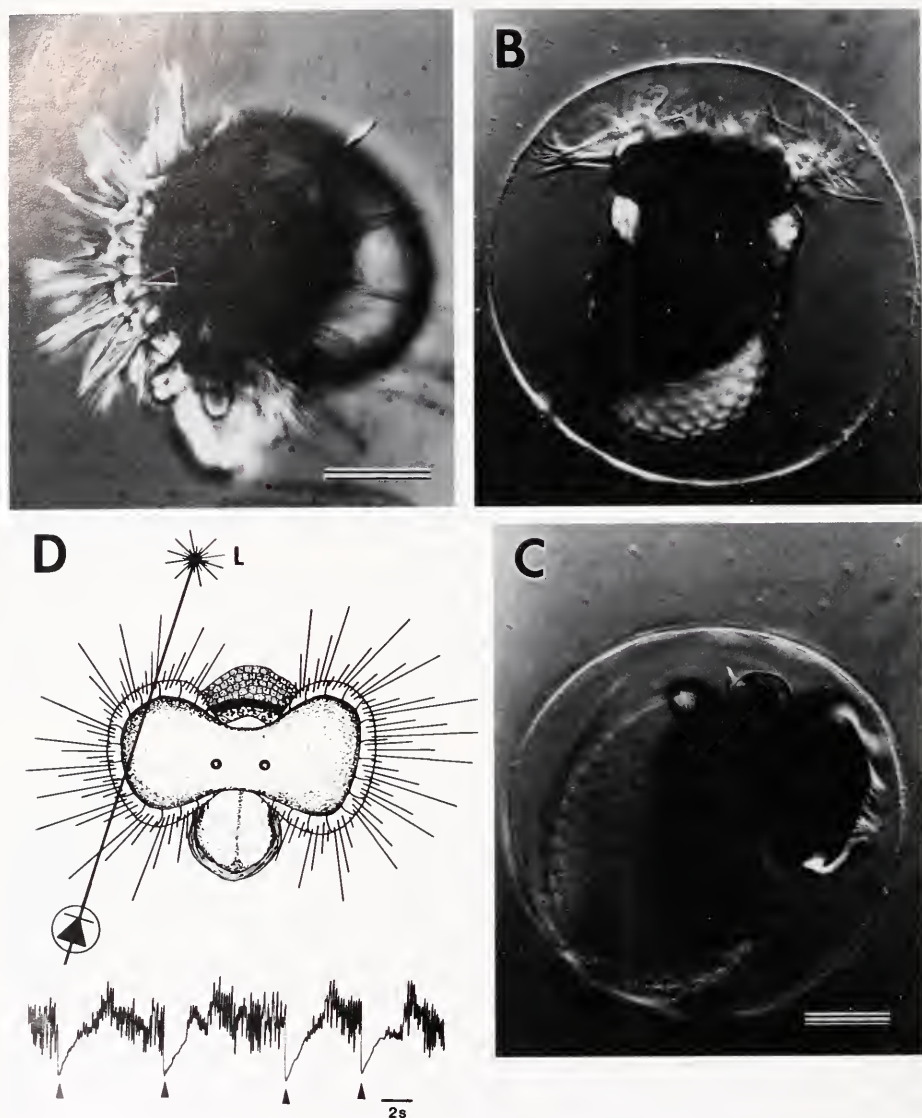


FIGURE 1 A-D. A. Free-swimming veliger of *Calliostoma ligatum* showing the laeoplectic metachronal waves of the pre-oral cilia. Waves move clockwise in this view. Note the outlines of individual pre-oral ciliated cells (arrow). Scale = 100 μ m. B. 96-hour-old veliger in egg capsule showing normal ciliary beating. C. Same larva as in B, but with cilia in the "arrested" position. Cilia are held in the characteristic conical tuft. Note that the foot and velum are still extended, but severe stimuli may cause their withdrawal into the shell. Scale for B and C = 100 μ m. Light micrographs were made using a Zeiss strobe flash. D. Laser arrangement used to monitor the frequency of metachronal waves and ciliary arrest. Laser beam is aimed at a photodiode and the veliger is positioned so that the cilia alternately bisect the beam. The large downward deflections in the voltage record (arrows) correspond to velum-wide ciliary arrests. The small oscillations between arrests represent the metachronal waves. L—laser.

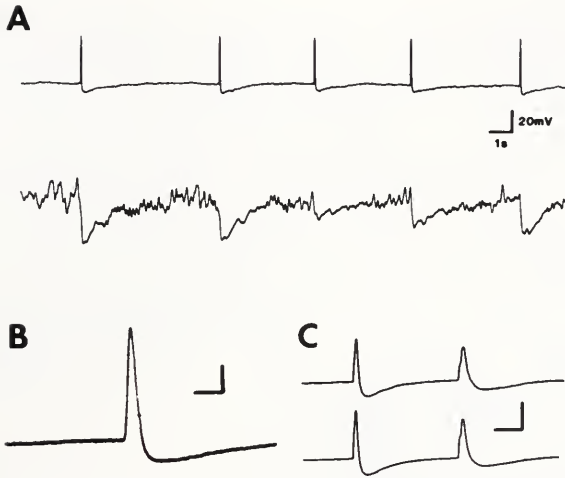


FIGURE 2 A-C. Intracellular electrode recordings from pre-oral ciliated cells of *Calliostoma ligatum* veligers. A. Single electrode recording from a ciliated cell (top) with a simultaneous photodiode voltage record (bottom) of ciliary beating and arrests. Note that each spike corresponds 1:1 to a large downward deflection of the diode voltage record, indicating a velum-wide ciliary arrest. The third spike also caused a velum-wide arrest, but the veliger had moved slightly to a position less than optimal for detecting ciliary arrest. Cilia gradually begin to beat within 1–2 s after a spike. Recommencement of ciliary beating is shown by the small oscillations which gradually increase in amplitude until the normal metachronal wave is re-established. B. Single oscilloscope sweep of a spontaneous, ciliated cell action potential. Note the absence of EPSP activity on the depolarizing phase. Horizontal scale = 50 ms, vertical scale = 10 mV. C. Dual electrode recording from opposite velar lobes, showing synchrony of action potentials. Note the distinct shoulder on the rising phase of the smaller amplitude second spike. Horizontal scale = 200 ms, vertical scale = 20 mV.

Physiology of pre-oral ciliated cells

Intracellular recordings from pre-oral ciliated cells show a resting potential of -60 mV. These cells exhibit no spiking activity or membrane potential oscillations during normal ciliary beating. However, coordinated, velum-wide arrests of pre-oral cilia correlate 1:1 with action potentials (Fig. 2A). These spikes have a positive phase lasting 40 ms, a peak amplitude of 45–50 mV, and a 10 mV hyperpolarizing undershoot lasting 700 ms (Fig. 2B). The hyperpolarizing phase is usually absent upon initial penetration of a cell, although spikes, lacking this phase, still cause ciliary arrest. Recordings from some ciliated cells show summing excitatory postsynaptic potentials (EPSPs) leading to an action potential, while others show no such EPSPs (Fig. 2B, C). The absence of EPSPs in some spikes may indicate that the synaptic site(s) is far enough away from the recording site such that EPSPs have decayed. We do not know if every pre-oral cell is innervated, but these results would suggest that they are not. Spontaneous spikes recorded from opposite velar lobes are well synchronized (Fig. 2C), showing about a 1 ms peak-to-peak delay. A stimulated spike propagates at about 20 cm/s. About 1–2 s after a spike, cilia slowly begin to beat again and the metachronal wave is re-established. However, at spiking frequencies greater than approximately 0.4 Hz, cilia remain arrested or twitching in the arrested position. Spike bursts rarely occurred at higher frequencies, but Figure 2C shows one such example. Note that the amplitude of the second spike in the burst is slightly reduced. The absolute refractory period for stimulated spikes is about 200 ms.

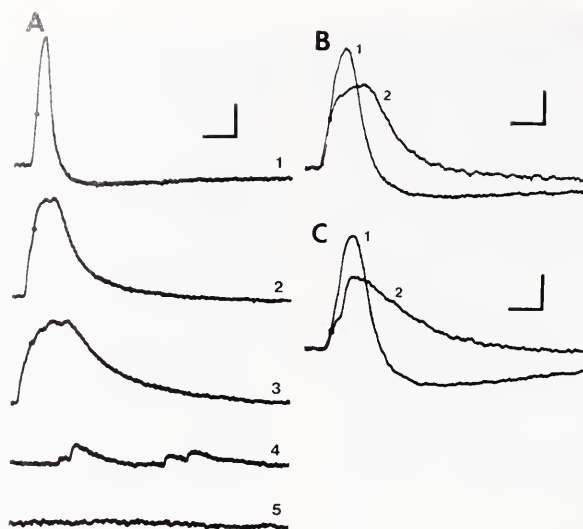


FIGURE 3 A. Intracellular electrode recording from pre-oral ciliated cells of *Calliostoma ligatum* veligers. Horizontal scale = 50 ms. (1, 2, 3) 200 ms (4, 5), vertical scale = 10 mV. 1. Single oscilloscope sweep of a spontaneous action potential in normal seawater. Note the short duration positive phase and long duration hyperpolarizing undershoot. 2. One minute after beginning replacement of normal CaCl_2 seawater with 10 mM CoCl_2 seawater. Note the loss of the hyperpolarized undershoot and the broadening of the spontaneous action potential. Spike still caused a velum wide ciliary arrest. 3. After two minutes, depolarization no longer caused velum-wide ciliary arrest, although localized "stutters" in ciliary beating were visible. 4. Three minutes in 10 mM CoCl_2 seawater, small spontaneous EPSPs are still present. 5. Five minutes in 10 mM CoCl_2 seawater. All synaptic activity is abolished. Spikes could not be initiated by intracellular stimulation. Upon return to normal seawater, spikes return as in 1. Same electrode penetration for 1-5. B. Spontaneous action potential of pre-oral cells in normal seawater (1) and about 5 minutes after the addition of 5 mM 4-aminopyridine (2). Horizontal scale = 20 ms, vertical scale = 10 mV. C. Action potential of pre-oral cells in normal seawater (1) and two minutes after the addition of 10 mM tetraethylammonium chloride. Horizontal scale = 20 ms, vertical scale = 10 mV.

Pre-oral ciliated cell action potentials are reversibly reduced in amplitude and are eventually abolished when CaCl_2 is replaced by an equimolar concentration of CoCl_2 (Fig. 3A). Cilia beat continuously and action potentials could not be elicited by intracellular electrode stimulation in 10 mM CoCl_2 seawater. Increasing concentrations of Mg^{++} produced a similar effect, but even at 120 mM Mg^{++} , a 5–10 mV depolarization was still detectable. It appears that Ca^{++} alone is responsible for the depolarization since a reduction of Na^+ to 25% of normal seawater (107.5 mM NaCl and 322.5 mM choline chloride) had no effect on spike amplitude or duration (not shown). The initial effect of the addition of Co^{++} on the spike is a broadening of the positive phase and a loss of the distinct hyperpolarizing undershoot (Fig. 3A). These effects suggest that Ca^{++} -dependent potassium currents are responsible for the hyperpolarization phase and these currents might be blocked by K^+ channel blockers (Hille, 1984). Addition of 5 mM 4-aminopyridine (4-AP) or 10 mM tetraethylammonium chloride (TEA) (Sigma) mimics the initial Co^{++} effects by eliminating the hyperpolarizing undershoot and broadening the spike (Fig. 3B, C).

Pre-oral ciliated cells are electrically coupled to each other. Depolarizing and hyperpolarizing current injected into one cell causes a proportional depolarization and hyperpolarization in neighboring cells (Fig. 4A). A brief pulse of suprathreshold positive current can produce a spike that propagates throughout the velum and causes

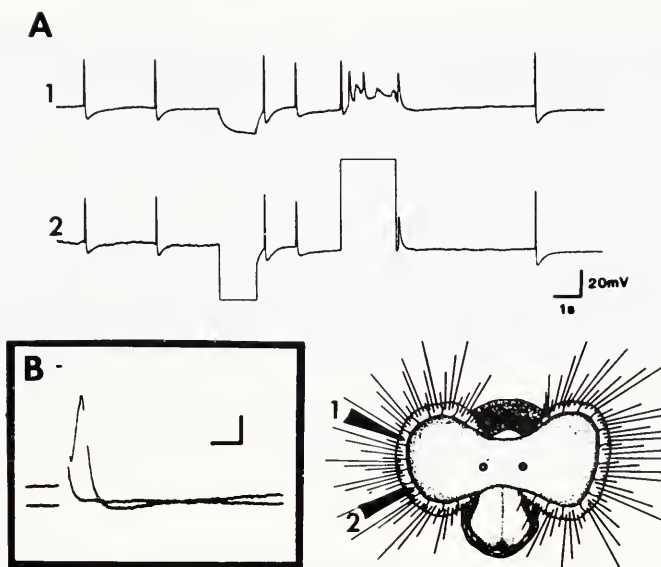


FIGURE 4 A, B. Intracellular electrode recordings from pre-oral ciliated cells of *Calliostoma ligatum* veligers. A. Simultaneous dual electrode recording demonstrating electrical coupling between ciliated cells. Hyper- and depolarizing current (± 5 nA) injected into electrode #2 causes a corresponding hyper- and depolarization of a neighboring ciliated cell (electrode #1). Similar results were observed when electrodes were located on opposite velar lobes. Note the synchrony and similarity of the spontaneous spikes in both recordings. Electrode bridge was not balanced. B. Successive sweeps of stimulated ciliated cell action potentials, using the same electrode penetration. In the first sweep, a brief subthreshold pulse of depolarizing current caused no spike. A spike was elicited in the second sweep when a suprathreshold current pulse was injected into the cell. Horizontal scale = 50 ms, vertical scale = 10 mV.

ciliary arrest (Fig. 4B). Ciliated cells are dye coupled to each other as Lucifer Yellow injected into one cell rapidly spreads throughout all pre-oral ciliated cells (Fig. 5).

We found that it was possible to modulate the inherent frequency of ciliary beating by injecting ramp depolarizing current into a ciliated cell. As the membrane potential of a ciliated cell is depolarized from resting potential, cilia of the impaled cell and those immediately adjacent to it begin to slow. As the cell is depolarized further, a wave front of slowing cilia spreads symmetrically about the electrode until all pre-oral cilia slow and eventually stop beating (Fig. 6a, c). If the stimulus current is turned off rapidly, cilia of all cells begin to beat again. However, if the current is slowly reduced back to zero, then the cilia far from the electrode begin to beat first, followed by those close to the electrode. From these results, it appeared that the frequency of pre-oral ciliary beating was dependent upon the level of depolarization of the ciliated cell membrane, although the laser arrangement shown in Figure 1D did not have the resolution to detect small changes in ciliary beat frequency that accompanied the slow depolarizations. We tried to document this phenomenon by making stroboscopic measurements of changes in ciliary beat frequency while depolarizing the pre-oral cells. This was done by increasing the concentration of KCl in the bath to depolarize the velum as a whole, rather than from a point source as in Figure 6A, C. Increases in the concentration of KCl to 0.5, 0.75, 1.0, 1.25, 1.75, 2.0 mM, above normal seawater linearly depolarizes the resting potential by 4, 6, 7, 8, 10, 11 mV, respectively. While depolarizing the membrane potential, however, the spiking frequency

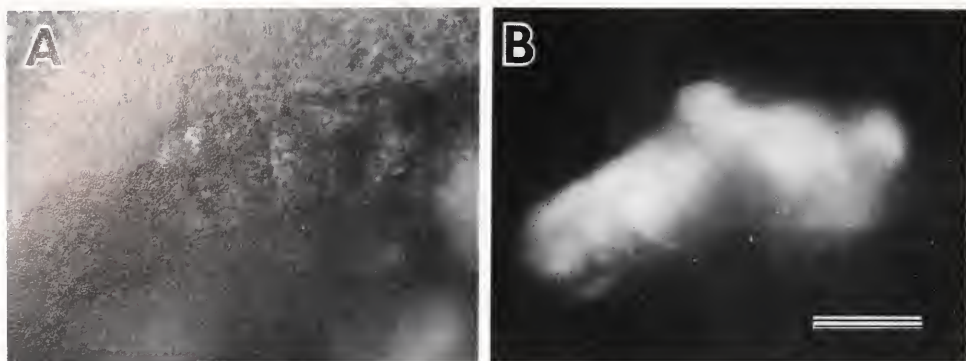


FIGURE 5 A, B. Lucifer Yellow dye coupling of pre-oral ciliated cells of live *Calliostoma ligatum* veligers. A. Brightfield micrograph of the velum. Cilia extend toward the top of the photo. B. Fluorescence micrograph of the same portion of the velum as A. A single cell was filled and dye spread throughout all cells. Note the outlines of individual cells. Scale = 30 μm .

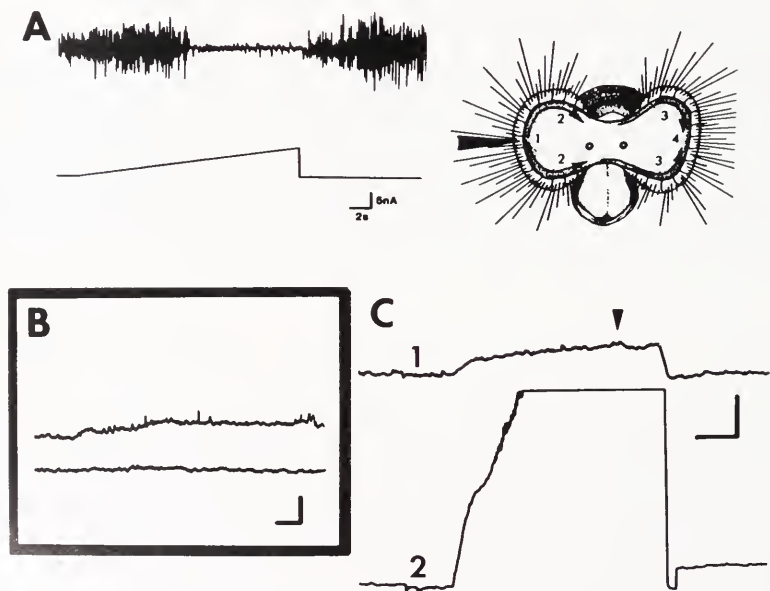


FIGURE 6 A-C. Intracellular electrode recordings from pre-oral ciliated cells of *Calliostoma ligatum* veligers. A. Ramp depolarizing current injected into a single ciliated cell causes an initial slowing of cilia at position 1. As the ramp continues, the wave front of slowing cilia follows the arrows and cilia at position 2 begin to slow when those around 1 have stopped. This wave front passes through 3 to 4, whereupon all cilia have stopped. Laser record (top) shows that when the current is terminated, cilia begin to beat again. The laser beam passed through position 3 and thus detected the arrest of cilia in this area alone. All cilia were arrested before the current ramp reached its maximum. B. Simultaneous dual electrode recording from ciliated cells on opposite velar lobes. Top trace shows a spontaneous slow depolarization with numerous high frequency EPSPs while bottom trace remains at resting potential. Horizontal scale = 1 s, vertical scale = 10 mV. C. Simultaneous dual electrode recording from ciliated cells on opposite velar lobes. Depolarizing current (up to 5 nA) was gradually injected into electrode #2. Electrode #1 shows the corresponding depolarization. Cilia began to slow during the depolarization and eventually arrested at the position of electrode #1 at the arrow. Electrode bridge was not balanced. Horizontal scale = 2 s, vertical scale = 20 mV.

increases and consequently the cilia remain arrested, or the metachronal wave was disrupted to the extent that visual synchronization was impossible. This fact hampered the visual measurement of ciliary beat frequency for a range of membrane potentials, and thus we were only able to document beat frequency reductions in response to small depolarizations. In one veliger, ciliary beat frequency in normal seawater was 7.2 Hz. This frequency was reduced by 13% to 6.3 Hz after increasing the concentration of KCl by 1.5 mM. This increase in KCl depolarized the -60 mV resting potential by 9 mV or 15%.

We have observed occasional spontaneous, high frequency EPSPs that are superimposed on long duration depolarizations (Fig. 6B). These slow depolarizations likely correspond to the general slowing of ciliary beating as described above, although we could not monitor ciliary beat frequency during dual intracellular electrode recordings. Slow depolarizations may be recorded on one velar lobe and not the other (Fig. 6B), whereas spikes are always observed velum-wide. These collective findings suggest, although admittedly do not prove unequivocally, that the frequency of pre-oral ciliary beating is dependent upon the depolarization level of the ciliated cell membrane and this level is modulated by excitatory synaptic input.

Morphology of pre-oral ciliated cells

The morphology of pre-oral ciliated cells of *C. ligatum* are very similar to those of *Mangelia* spp., which have been well described by Mackie *et al.* (1976). Thus, only a brief description of these velar cells of *C. ligatum* will be given here. Pre-oral ciliated cells are characterized by the presence of numerous scythe-like compound cilia (Fig. 7A). There are usually about 25 of these cilia per cell and each one is composed of approximately 50 individual cilia. Each individual cilium is anchored in the cell by a distinct basal body and a long striated rootlet (Fig. 7B). The rootlets are robust and extend well into the cell. Numerous mitochondria are concentrated just below the basal bodies. Another distinct feature of these cells is the presence of numerous, large lipid vacuoles, concentrated near the base of the cell (Fig. 7A, D). These vacuoles appear to be similar to the "reserve food vacuoles" described by Carter (1928). Distinctively less dense sheath cells lie on the oral and aboral sides of ciliated cells as in *Mangelia* spp. (Mackie *et al.*, 1976). Sheath cells bear numerous microvilli and only a few simple cilia. Lipid vacuoles are conspicuously absent from sheath cells. Pre-oral ciliated cells are connected to each other by numerous gap junctions and a few septate demosomes (Fig. 7B, C). Ciliated cells are richly innervated by axons containing electron-lucent vesicles (Fig. 7D). Most of these neuro-ciliary synapses are found at the proximal end of the cells, although we have observed some distally.

DISCUSSION

We have provided evidence to suggest that the beating frequency of the locomotory, pre-oral cilia of competent veligers of *C. ligatum* is dependent upon the level of depolarization of the ciliated cells' membrane and that this level is modulated by excitatory neuronal input. Pre-oral cilia appear to have an inherent beating frequency which is seen at resting potential, or when the ciliated cells are isolated from excitatory inputs either physically by dissociation or chemically in high Mg^{++} solutions. The inherent beating frequency is reduced by excitatory inputs, presumably originating from the cerebral ganglion. This ganglion is highly developed in early veliger stages of *Aplysia californica* (Kandel *et al.*, 1980) and its axons have been shown to innervate the velar ciliated cells in several veligers (Carter, 1926, 1928; Mackie *et al.*, 1976).

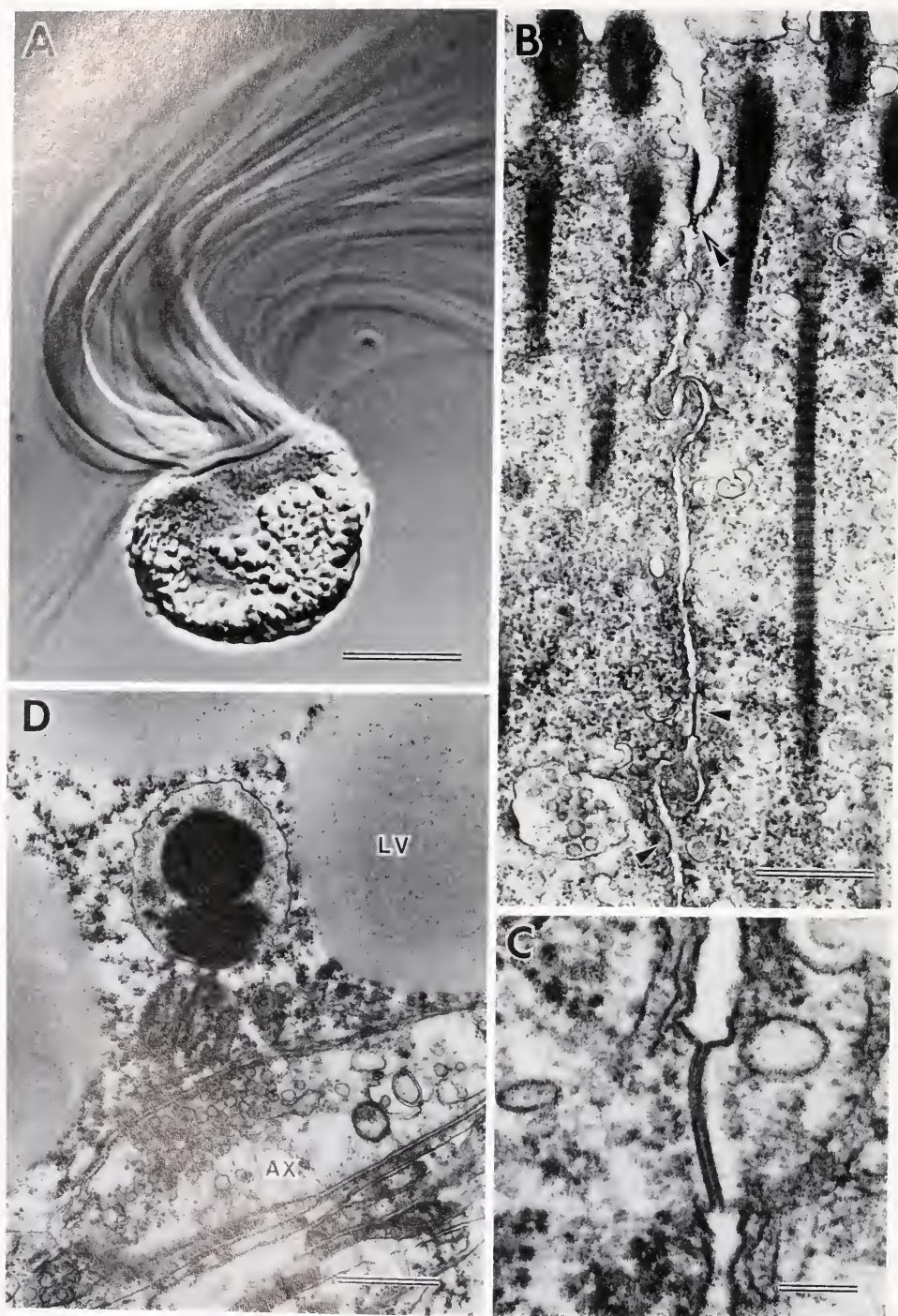


FIGURE 7 A-D. A. Side view of a single, isolated pre-oral ciliated cell from *Calliostoma ligatum*. Aboral end is to the right, oral end is to the left. Effective stroke is from left to right. Note the scythe-like compound cilia which lie in rows orthogonal to the direction of beat. Prominent spheres at the base of the

We also find many neuro-ciliary synapses (Fig. 7D), but we have not determined the origin of these axons, nor do we know if all pre-oral cells are innervated.

Control of locomotion

Veligers are capable of modulating the inherent frequency of ciliary beating to yield two distinct types of locomotory behavior. In the first type, normal ciliary beating is periodically interrupted by rapid, coordinated, velum-wide ciliary arrest (Figs. 1D, 2A). Coordinated velum-wide ciliary arrests are caused by a ciliated cell action potential, which results from summing EPSPs. This spike propagates rapidly throughout the electrically coupled pre-oral cells (Fig. 2C). Cilia remain arrested with spiking frequencies of 0.4 Hz or greater. One to two seconds after the spike, cilia begin to beat and during normal ciliary beating, the membrane potential remains at resting potential and little synaptic activity is observed. This modulation of ciliary beating is similar to that in other veligers (Mackie *et al.*, 1976) and other metazoan ciliated systems (Mackie *et al.*, 1974; Saimi *et al.*, 1983a; Arkett, 1987) where a rapid stoppage of ciliary beating is advantageous (see below).

We have provided several pieces of evidence to suggest that the action potential of the pre-oral ciliated cells is dependent upon an influx of Ca^{++} . Replacement of Ca^{++} by Co^{++} reduces the amplitude and eventually blocks the spike and prevents ciliary arrest. (Fig. 3A). This finding corroborates the study by Korobtsov and Sakharov (1971) which showed that Co^{++} blocks ciliary arrest behavior. We have also shown that the K^+ channel blockers, 4-AP and TEA, eliminate the long hyperpolarizing undershoot and broaden the spike, suggesting Ca^{++} -dependent K^+ channels are present in the pre-oral cells. After the large influx of Ca^{++} during the depolarizing phase of the spike, these K^+ channels may be activated, thereby hyperpolarizing the cell and preventing further Ca^{++} entry (Hille, 1984). As the internal Ca^{++} concentration is reduced, cilia may resume beating and a second spike may be initiated. These large ciliated cells should prove to be a valuable preparation for more fully characterizing the currents involved in ciliary arrest and the resumption of beating.

The second type of locomotion is produced by a gradual slowing of ciliary beating, rather than by full velum-wide ciliary arrests. High frequency EPSPs generate long duration, slow depolarizations of the ciliated cells (Fig. 6B, C). As the ciliated cells gradually depolarize, the frequency of ciliary beating appears to drop. We have not observed veligers of this species to use this method of modulating ciliary beating frequency to stop ciliary beating, only to slow it. Localized excitatory input to pre-oral cells can alter the ciliary beating frequency of one velar lobe and not the other (Fig. 6B). This differential ciliary beating may be used by the veliger in conjunction with velar muscle contractions to make turns in the swimming path.

We have demonstrated that an action potential is actively propagated throughout the ciliated pre-oral velar epithelium. The rapid depolarizations associated with ciliary arrests in ascidian branchial basket (Mackie *et al.*, 1974; Arkett, 1987) and *Mytilus* gill (Murakami and Machemer, 1982; Saimi *et al.*, 1983a) appear to be conducted

cell are lipid vacuoles. Scale = 25 μm . B. Electron micrograph of the distal portion of two ciliated cells connected by gap junctions (single arrows) and a septate desmosome (double arrow). Note the robust striated rootlets. Scale = 0.5 μm . C. A single gap junction connecting two pre-oral ciliated cells. Scale = 0.1 μm . D. An axon (AX) containing electron lucent vesicles at the base of a ciliated cell. LV—lipid vacuole; Scale = 0.5 μm .

passively through gap junctions connecting ciliated cells. We have also provided evidence that suggests a dependence of ciliary beat frequency on the level of depolarization of the ciliated velar cells, although unequivocal demonstration of this point was not possible using the intact veliger. The mechanisms for *Mytilus* gill appear to differ since Saimi *et al.* (1983b) stated that the ciliary beat frequency is independent of the ciliated cell membrane potential. Yet, Aiello *et al.* (1986) showed that dopamine caused a dose-dependent decrease in the endogenous ciliary beating frequency of *Mytilus* lateral cilia, but they did not monitor ciliated cell membrane potential. In *Paramecium*, forward swimming increases as the membrane potential becomes more hyperpolarized (Machemer, 1974; Bonini *et al.*, 1986). We found that hyperpolarization of veliger pre-oral ciliated cells has no apparent effect on beating frequency. Pre-oral cilia never appear to beat faster than their inherent frequency.

Importance of electrically coupled ciliated cells

The pre-oral ciliated cells are electrically coupled to each other (Figs. 4A, 5) by numerous gap junctions between the cells (Fig. 7B, C). Strong electrical coupling should ensure that EPSPs will summate spatially from velum-wide excitatory input to ciliated cells and temporally due to a reduction in the junctional shunting of current between the cells and a longer time constant (Getting, 1974; Berry and Pentreath, 1977). These features lead to a regenerative action potential in the pre-oral cells. Electrical coupling of these cells should ensure that an action potential generated at one point on the velum will propagate rapidly throughout the pre-oral cells, thereby synchronizing the arrest of all pre-oral cilia (Figs. 2C, 4A, B). This property of rapid junctional transmission is one of the most important features of electrically coupled systems (Bennett, 1977). However, we cannot rule out an alternative explanation, namely that ciliary arrest in the two velar lobes is synchronized by bilaterally symmetric pairs of neurons in the cerebral ganglion.

The electrical coupling properties of pre-oral cells may also make possible small, local changes in the membrane potential without spikes. Local CNS input may depolarize a portion of the velum, but because the input resistance of the velar cells is low, current is shunted throughout the pre-oral cells and no spikes are generated. However, such local input can result in long duration, slow depolarizations (Fig. 6B, C) and thus a differential slowing of ciliary beat frequency on different sides of the velum.

Functional significance of locomotory control

We have demonstrated that the veligers of *C. ligatum* are capable of controlling locomotory behavior. This control appears to be expressed in two different types of locomotion. The alternating upward swimming and sinking upon ciliary arrest is probably the best known type of locomotion and it has been proposed as a mechanism for regulating position in the water column (Richter, 1973; Cragg and Gruffydd, 1975; Hidu and Haskin, 1978). Net upward movement could be accomplished by decreasing the frequency or duration of ciliary arrests, consequently increasing the amount of time spent swimming. Since veligers generally sink faster than their maximum ascent speed (Cragg, 1980; Chia *et al.*, 1984), an increase in the frequency of ciliary arrest should result in a net downward movement. Although the main use of this type of locomotion may be in regulating diel vertical migration patterns (Richter, 1973), it may also be important as a defence mechanism. Contact with "foreign" objects or potential predators might cause a rapid ciliary arrest and sometimes a retraction of the body into the shell, whereupon the larva sinks rapidly (Fretter, 1967).

The second type of locomotion is characterized by more subtle changes in normal ciliary beating. The gradual, velum-wide slowing of locomotory cilia without full arrest would enable the veliger to either swim slowly upward or sink at a slower rate than during full ciliary arrest (Richter, 1973; Cragg and Gruffydd, 1975). This behavior appears to be closely linked to the veliger's satiation level (Fretter and Montgomery, 1968) and may enable feeding veligers to remain within a zone of abundant food. The differential ciliary beating of velar lobes observed in *C. ligatum* in conjunction with muscle contraction of the velum may enable the veliger to vary its swimming direction.

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FEEDING BEHAVIOR IN *HYDRA*. I. EFFECTS OF *ARTEMIA* HOMOGENATE ON NEMATOCYST DISCHARGE*

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ABSTRACT

Inhibition of desmoneme and stenotele nematocyst discharge occurs when *Hydra attenuata* are fed to repletion. Inhibition can be induced by the application of prey homogenates in the external medium. The onset of inhibition is relatively rapid (<30 s) while the release from inhibition is much slower (>20 min). Inhibition is concentration-dependent. Gel chromatography separation of homogenate shows that the inhibitory substance(s) have a molecular weight greater than 5000. These substances cause the strongest stenotele inhibition and are least effective in activating the feeding reflex (mouth opening and tentacle concerts) which is caused by smaller molecular weight substances. The receptor sites for the inhibitory substance(s) are located on the external surface of the hydra tentacle. Accumulation of prey substances may be the mechanism by which stenotele discharge is inhibited when hydra are fed to repletion.

INTRODUCTION

Nematocysts are highly specialized organelles that serve several functions in coelenterates. Nematocysts are used to help capture and kill prey. In some coelenterates, nematocysts are also necessary for locomotion and defense (Picken and Skaer, 1966; Mariscal, 1974).

In *Hydra* two types of nematocysts are involved with the feeding response. Desmonemes function in prey capture and have a tightly wound thread which wraps around the prey. Stenoteles have a penetrant shaft and function as killing nematocysts by piercing the prey and releasing a lethal toxin (Ewer, 1947). When hydra are fed to repletion, however, they lose their ability to discharge nematocysts. Several causes for nematocyst inhibition have been proposed. (1) *Hydra* become less responsive to stimulation and fewer stenoteles are brought to bear against the prey (Burnett *et al.*, 1961). (2) Either a metabolite from the prey or a product of digestion inhibits nematocyst discharge (Smith *et al.*, 1974). (3) A factor from the nematocysts themselves inhibits nematocyst discharge (Ruch and Cook, 1984).

We now present evidence that the discharge of stenotele nematocysts can be inhibited by the external application of prey substances and that the receptor sites for these substances are located on the tentacle.

MATERIALS AND METHODS

Specimens of *Hydra attenuata* from a single asexually reproducing clone were used in all experiments (Kass-Simon and Potter, 1971). They were raised in BVC

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solution (Loomis and Lenhoff, 1956), and were fed daily from a culture of *Artemia* nauplii (brine shrimp).

Brine shrimp homogenates were prepared by concentrating large quantities of brine shrimp nauplii in a filter (Whatman #4 paper) and washing them thoroughly with distilled water. The nauplii were drawn off with a syringe and the concentrate was repeatedly forced through the bore of an 18 gauge needle until its consistency was smooth. This crude homogenate was centrifuged in a clinical centrifuge ($\sim 1000 \times g$, 5 min). The water-soluble middle layer was drawn off and recentrifuged to complete the removal of insoluble material. The finished homogenate was then divided into samples of 1 to 3 ml and frozen at -5°C until use.

The relative concentrations of various homogenates were determined by collecting 1 ml subsamples of brine shrimp from the shrimp collected from the mass cultures. These were counted and the number of brine shrimp per ml computed.

Stenotele discharge was defined as being inhibited when a hydra rejected, in succession, five offered brine shrimp that made contact with the tentacles and that were still swimming normally after 30 s (Smith *et al.*, 1974). Brine shrimp were introduced into the medium surrounding the hydra with a pasteur pipet and were directed towards the tentacles with forceps. To be classified as having made contact, brine shrimp must have distinctly moved a tentacle. Slight brushes with the tentacle were difficult to discern and were not counted. Hydra were transferred from dish to dish using large bore dropping pipets.

Brine shrimp killing was grouped into two categories: (1) brine shrimp that were captured and killed, and (2) those that made contact but were not captured or killed. Brine shrimp which were initially captured, but then escaped were classified in group 2.

Feeding hydra to repletion

Hydra were placed in 10 ml of BVC solution in a 60×15 mm glass petri dish, and were offered 2 brine shrimp every 5 minutes for 150 min (30 trials). During the experiment the number of brine shrimp captured, killed, and ingested was monitored. If brine shrimp made contact with the tentacles and were not captured, brine shrimp continued to be offered until either two brine shrimp were captured or a total of five offerings were made.

Inhibition of stenotele discharge

Hydra were placed in 5 ml of 1/25 dilutions of brine shrimp homogenates and were tested for inhibition of stenotele discharge as above. Animals which displayed significant inhibition of stenotele discharge were then transferred to fresh BVC solution for 1 min. They were again transferred to fresh BVC solution for 45 min and again tested with 5 brine shrimp. Control animals were placed in 5 ml of BVC solution and tested for stenotele discharge after 5 min.

Effects of homogenate on stenotele discharge

Two sets of experiments were performed to test the effects of homogenate on nematocyst discharge. Hydra tentacles were ablated 24 h prior to testing. They were then placed in 1/25 dilutions (0.74 mg/ml protein) of brine shrimp homogenates. First, individual tentacles were placed on a glass slide in the diluted homogenate for 5 min and covered with a coverslip; the number of discharged nematocysts were counted under $400\times$ magnification. An equal number of control tentacles were

placed in BVC solution. In addition, tentacles in diluted homogenate were placed in a 0.15×1.0 cm cell and the number of discharged nematocysts were counted after 5 and 25 min while being observed under an inverted microscope (400 $\times$). Control tentacles were placed in BVC solution.

Time course of inhibition and dose response

The onset of inhibition was determined by placing individual hydra in 1/25 dilutions of brine shrimp homogenate. Hydra were transferred into the homogenate and tested with five brine shrimp at given time intervals after transfer.

To test for recovery from inhibition, several hydra were placed in 1/25 dilutions of homogenate for 5 min, rinsed with fresh BVC solution, and then rapidly transferred into separate dishes containing 5 ml of BVC solution. They were then tested with 5 brine shrimp at regular time intervals ranging from 2.5 min to 22.5 min.

To determine the concentration of homogenate required for inhibition, the homogenate was diluted serially by factors of 10, resulting in dilutions of 10^{-1} to 10^{-6} . Individual hydra were placed in 5 ml of diluted homogenate for 5 min and were then tested with 5 brine shrimp. The protein concentration of the homogenate was determined by measuring the protein concentrations of an homogenate from the same batch (Lowry *et al.*, 1951).

Extraction of inhibitory substances

Gel filtration chromatography (Determan, 1969) was used to fractionate the homogenate. A 15.5 by 1.5 cm column with a polystyrene bed support was packed with sephadex G-25-80 beads (Sigma Chemical Co.). Solvent (BVC solution) was placed in a reservoir raised several feet above the column to create a slight positive pressure. A 1-ml sample of homogenate was used for each experimental run. Sample collection began 50 drops prior to the first appearance of homogenate fractions from the column. Fifteen 20-drop samples were collected. The first appearance of homogenate fractions from the column could be clearly observed due to the cloudiness of the fraction and the slight orange color. This correlated closely with calculations of protein elution from Determann (1969).

Each of the 15 fractions was diluted by 1/20 with BVC solution. One hydra was placed in 5 ml of diluted fraction for 5 min. The level of stenotele discharge, mouth opening, and tentacle activity was determined. Numerical values, as given in Table I, were assigned to each of the responses for data analysis. In a separate set of experiments, 5×10^{-4} M reduced glutathione (GSH, Sigma) was run through the column to locate the fractions in which substances that activate the feeding response would be found (Loomis, 1955).

Localization of receptors

To test for receptors in the gastrovascular cavity, injections of homogenate were made through the basal pore. The injection apparatus was composed of drawn out polyethylene tubing attached to a Hamilton 10 microliter syringe (Smith *et al.*, 1974).

First, hydra were injected with 1.0 μ l of whole homogenate, and tested with 2 brine shrimp every 5 min for 35 min. At the end of 35 min additional homogenate was injected until it could be seen leaking from the hypostome. Two minutes later the hydra were again tested for stenotele discharge using 5 brine shrimp. As a control, 1.5 μ l of air was injected. Air bubbles remain intact and can easily be seen in the gut. After 5 min the hydra were tested for stenotele discharge with 5 brine shrimp.

TABLE I

Numerical values assigned to specific aspects of the feeding behavior in hydra: mouth opening and tentacle activity

Mouth opening response:	Numerical value:
Hypostome protruding from tentacle ring. Slight opening.	1
Moderate mouth opening. Mouth clearly open.	2
Large mouth opening, hydra may be inverting itself.	3
Tentacle activity:	
Slight activity, periodic concerts.	1
Continuous concerts.	2
Tentacle writhing.	3
Tight writhing, tentacles may have entered mouth.	4

Partial values given for activities between the defined steps.

In another series of experiments, hypostomes with attached tentacles (ablated 24 h earlier) were placed in 0.25 ml of BVC solution containing 10 μ l of homogenate. The animals were allowed 5 min to relax and were tested with 5 brine shrimp. Control animals were placed in BVC solution only. In addition, isolated tentacles were tested by the same procedure.

Different regions of a given tentacle were also tested for inhibition of stenotele discharge. A small quantity of homogenate was applied to the proximal half of a tentacle using a 10 μ l syringe. The extent of the cloud of homogenate could clearly be seen. The proximal half of the tentacle was kept in the homogenate for 2 min and then relocated to a clear region of the depression slide. Brine shrimp were added to the culture medium and the number of random contacts or kills were recorded for 2 min for each half of the tentacle.

If the proximal half of the tentacle became inhibited, that portion was ablated from the whole tentacle and placed in fresh BVC solution. It was allowed 1 h to recover and was then retested for stenotele discharge as above.

Statistical analyses were done on the University of Rhode Island computer system with software from Statistical Analysis Systems (Box 800, Cary, North Carolina). Where applicable, Wilcoxon Rank Sums, Kruskal-Wallis K Samples, and Linear Regression tests were used. Paired data were analyzed using the Wilcoxon Signed-Rank Test (Lentner, 1975).

RESULTS

Our observations with respect to hydra's behavior when fed to repletion (Fig. 1) essentially confirm the results of Smith *et al.* (1974) and Ruch and Cook (1984). The hydra readily killed the brine shrimp offered to them (trials 1–10). However, as additional shrimp were added to the hydra, the number of brine shrimp killed decreased. When the killing of brine shrimp began to decrease (trial #11) the hydra had killed 169 out of 170 brine shrimp (>99%). From trial #11 through #30 killing was reduced to <30% (89/320 brine shrimp). Ingestion was also reduced; 92% of the brine shrimp killed in the first 10 trials were ingested, whereas in the latter trials <40% were ingested.

During the latter trials the killing of brine shrimp by individual hydra were quite variable. A given hydra could be completely inhibited for two or more periods and

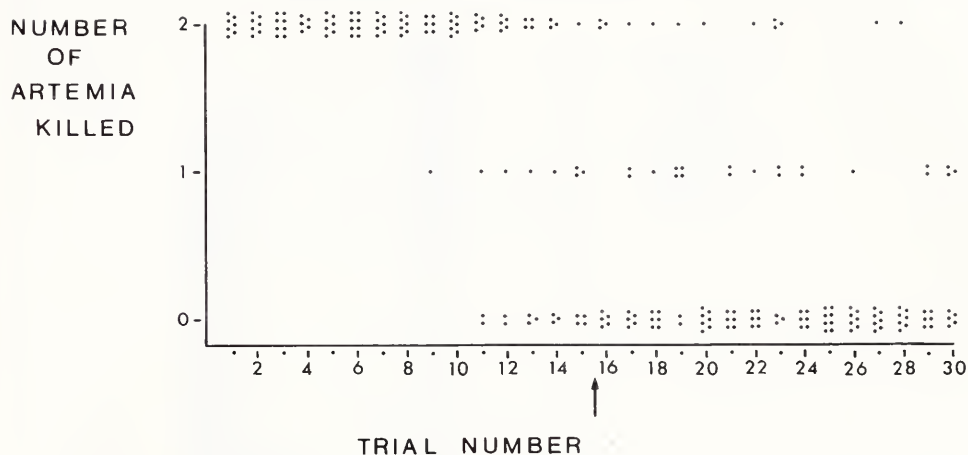


FIGURE 1. Number of brine shrimp killed/hydra when hydra were offered 2 brine shrimp at 5-min intervals for 30 trials. Arrow indicates the mean trial in which column contractions began. $n = 8$.

then kill one or two brine shrimp. Sometimes, brine shrimp that were captured were not killed. These would eventually pull free from the tentacles and continue swimming normally.

Application of brine shrimp homogenate (1/25 dilution) to the medium surrounding a hydra caused mouth opening and tentacle writhing responses. In these cases, when brine shrimp are placed near the tentacles of animals in homogenate, there is a significant decrease in the killing response compared to control animals in BVC solution ($P = .001$, Wilcoxon Rank Sums, Fig. 2). After a washing out period in which the hydra are placed in clean BVC solution, stenotele discharge returned and was (T_2 , Fig. 2) significantly greater than that in the test animals (T_1 , Fig. 2) ($P < 0.01$ Wilcoxon Signed-Rank).

Microscopic examination of hydra tentacles indicate that 1/25 dilutions of brine shrimp homogenates do not stimulate nematocysts to discharge. In both sets of experiments, test tentacles (dilute homogenate) were not significantly different from control tentacles (BVC solution). The five tentacles placed in homogenate for 5 min and covered with a coverslip produced no discharged nematocysts. Among control tentacles, one tentacle discharged a single stenotele. Among tentacles observed on an inverted microscope with no coverslip for 5 min, 3 out of 10 in homogenate discharged stenoteles (mean = 2.33 stenoteles/tentacle). Among 10 control tentacles, 4 discharged stenoteles (mean = 3.25 stenoteles/tentacle) and one discharged a desmoneme. No additional discharge was found at the 25 min interval. The results indicate that homogenate alone does not induce nematocyst discharge.

Almost complete inhibition appears to occur within 30 s of placing a hydra in diluted homogenate. No significant differences were found among the 5 tested time periods (30 s, 1, 2, 3, and 4 min.), indicating that the onset for inhibition was less than 30 s. Release from inhibition occurred in 20 min, after which the killing was restored to normal levels (Fig. 3).

In serial dilutions of homogenate, a linear relationship ($r = 0.82$) was found between the concentration of homogenate and the number of brine shrimp killed (Fig. 4). Although the number of brine shrimp killed by individual hydra varied, as a group

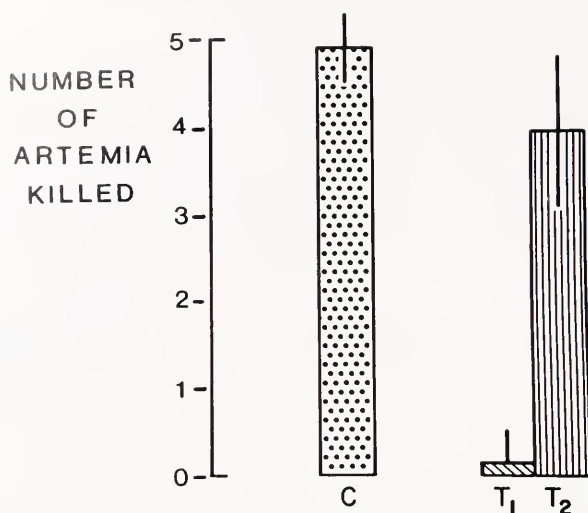


FIGURE 2. Number of brine shrimp killed (mean $\pm$ SD) during external application of 1/25 dilutions of homogenate. Each hydra was tested with five brine shrimp. C = Controls, in BVC solution; T₁ = tests, in dilute homogenate; T₂ = retests, retested after 45 min in BVC solution. $n = 6$.

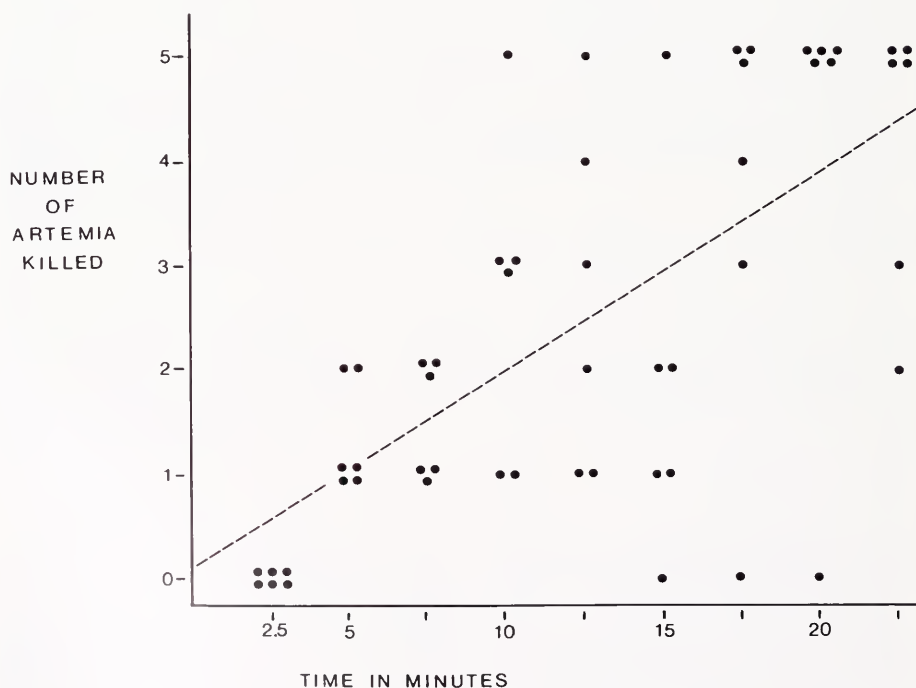


FIGURE 3. Release of stenotele nematocysts from inhibition. Hydra were placed in 1/25 dilutions of homogenate for 5 min and then transferred to fresh BVC solution. They were tested with five brine shrimp at given time intervals. $n = 8$, $r = 0.67$.

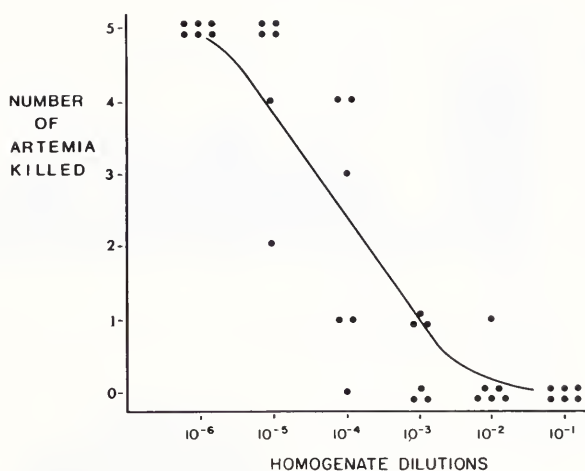


FIGURE 4. Serial dilutions of homogenate (18 mg/ml) that induce inhibition of stenotele discharge. Hydra were placed in given dilutions of homogenates for 5 min and then tested with 5 brine shrimp. $n = 6$. A linear regression was calculated between 10^{-5} and 10^{-2} , $r = 0.82$.

there was a 50% reduction in killing at a 10^{-4} dilution of homogenate. The protein concentration of the undiluted homogenate was 18 mg/ml. At 50% inhibition the protein concentration of the diluted sample was $1.8 \mu\text{g/ml}$.

Brine shrimp homogenates separated according to molecular weight were tested for their effects on stenotele discharge and for various aspects of the feeding response (Fig. 5). Significant differences were found between the killing of brine shrimp in different fractions ($P = .0001$, Kruskal-Wallis). The differences were between the first two fractions (control) and the largest molecular weight fractions, 3 and 4 (Multiple Comparisons based on Kruskal-Wallis). The other samples did not show a significant loss in stenotele discharge compared to the controls.

There were also significant differences in the mouth opening response between the different fractions ($P = .0001$, Kruskal-Wallis). Fractions 8 through 11 had a significantly higher mouth opening response. Fractions 3 and 4 (the large mol. wt. fractions) caused little or no response. Significant differences were also found for tentacle activity ($P = .0001$, Kruskal-Wallis). Fractions 8 through 12 caused significantly more activity than the other samples.

GSH ($5 \times 10^{-4} M$) was also run through the column. The results indicate that GSH had no effect on brine shrimp killing. Samples 7 through 11 produced at least $\frac{1}{2}$ maximal mouth opening response (Table I). Tentacle activity responses were at least $\frac{1}{2}$ maximal in samples 6 through 11 (Fig. 5). Little or no activity was found in samples 3 and 4 for either mouth opening response or tentacle activity.

Injections of whole homogenate into the gastrovascular cavity had no effect on stenotele discharge (Fig. 6). One hundred percent of the brine shrimp were killed during the seven testing periods. There was no indication of mouth opening or tentacle activity.

Only when homogenate leaked out of the hypostome (Fig. 6, T_2) was there a significant inhibition of stenotele discharge compared to control animals ($P = .009$, Wilcoxon Rank Sums). Leakage of homogenate can be caused by injecting excess amounts into the gastrovascular cavity and can be readily observed as a murky cloud

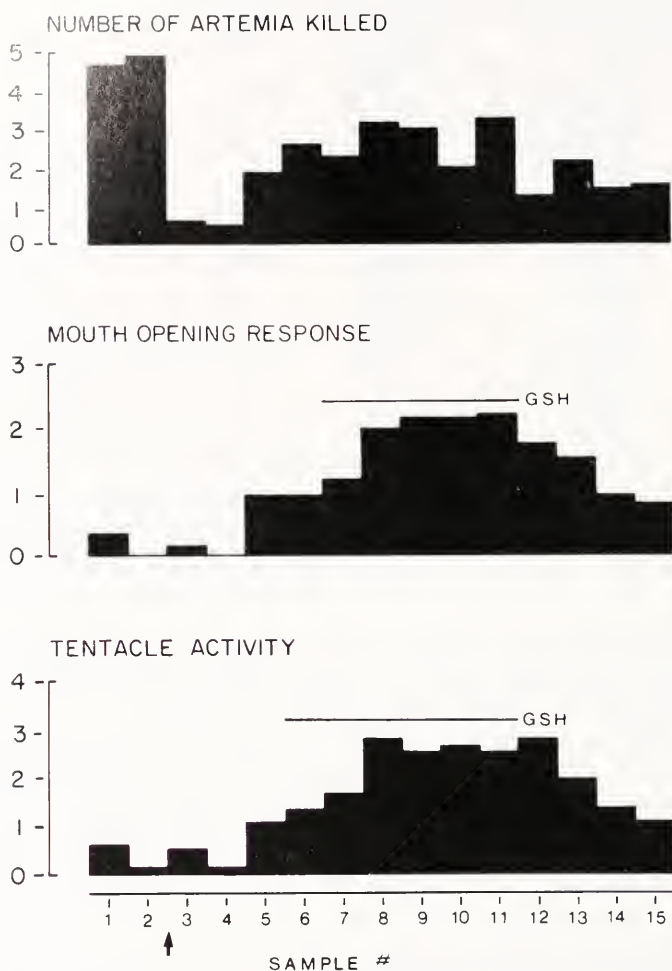


FIGURE 5. Homogenate separation using gel filtration chromatography. Values in each scale are defined in the methods section. The GSH lines indicate the samples with at least $\frac{1}{2}$ maximal response to 5×10^{-4} reduced glutathione. Arrow indicates the beginning of the fractionated homogenate in the samples. $n = 7$.

around the tentacles. When this occurred, mouth opening responses and tentacle concerts or writhing were also observed.

Neither columns nor hypostomes are required for stenotele discharge to be inhibited (Fig. 7). Both ablated hypostomes with tentacles and isolated tentacles displayed significant inhibition of stenotele discharge compared to controls ($P = .001$, both experiments, Wilcoxon Rank Sums). Similar results were found by Smith *et al.* (1974) and Ruch and Cook (1984) using hypostomes and tentacles.

Treatment of the base of tentacles with homogenate (Fig. 8, T_1) resulted in a significant loss of stenotele discharge compared to the tentacle base of control animals ($P = .003$, Wilcoxon Rank Sums). Brine shrimp killing for the tips of the tentacles, which had not been treated, were not significantly different from their corresponding controls. After placing the base of the tentacles in fresh BVC solution for 1 h, stenotele

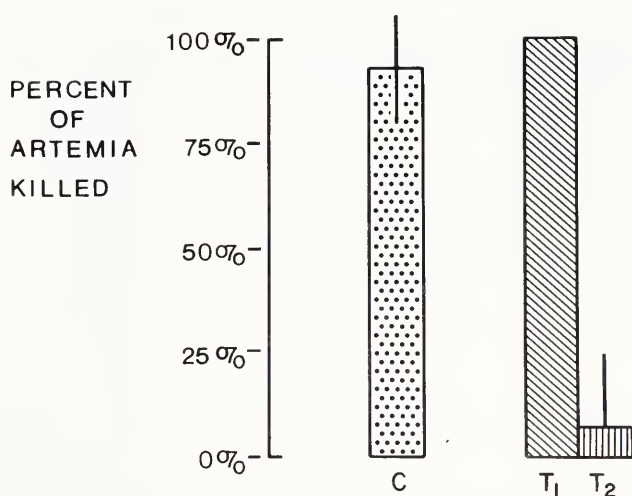


FIGURE 6. Injections of homogenate into the gastrovascular cavity through the basal pore. Control animals were injected with air. Results expressed as % brine shrimp killed (mean $\pm$ SD) by the tentacles. Percents were used since the total number of brine shrimp used varied in each test (T_1 = 14 brine shrimp, T_2 = 5). C = Controls, air injections; T_1 = test, homogenate injections; T_2 = retests, homogenate leaking out of the gastrovascular cavity. n = 5.

discharge in the base of the tentacles (Fig. 8, T_2) largely returned and was significantly different from the base of the test tentacles ($P < 0.01$ Wilcoxon Signed-Rank).

DISCUSSION

Stenotele discharge in hydra becomes inhibited when animals are fed to repletion. After repletion and the onset of inhibition, the animals remain at least partially inhibited until the end of the experiment (95 min). This compares favorably with results by Smith *et al.* (1974) which indicate that stenotele discharge remains partially inhibited until regurgitation (over 4 h).

Externally applied brine shrimp homogenates produced a rapid onset of inhibition of stenotele discharge (<30 s). Since the methods used to measure the time of onset were not sensitive enough to resolve times less than 30 s, a more exact time for onset of inhibition could not be determined. The release from inhibition is relatively slow. These findings also agree with work by Smith *et al.*, (1974) in which the hypostome and tentacles were removed from inhibited animals and placed in fresh culture solution; the killing response returned in 30 min.

The inhibitory effects of the homogenates were dependent upon concentration. At 50% inhibition of stenotele discharge, the concentration of homogenate was equal to the water soluble layer of 0.043 brine shrimp/ μ l (1.8 μ g/ml protein). This is a very small amount compared to the average number ingested by experimental animals (mean = 32 brine shrimp/hydra). Although the number of shrimp necessary to induce 50% inhibition of stenotele discharge is greater than the average number ingested by a hydra, it is probably incorrect to calculate the concentration of the inhibitory substances, surrounding a repleted and inhibited hydra, as though they were dissolved in the entire experimental dish. Loomis (1964) and Lenhoff (1965) showed that a concentration gradient does exist in the microenvironment surrounding a hy-

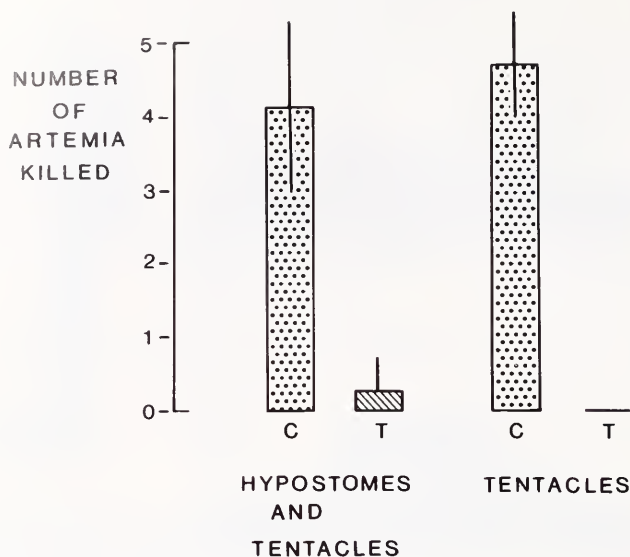


FIGURE 7. Inhibition of stenotele discharge in hypostomes and tentacles of hydra. Ablated hypostomes with tentacles ($n = 8$) and isolated tentacles ($n = 9$) were placed in $1/25$ dilutions of homogenate for 5 minutes and tested with 5 brine shrimp. C = controls, in BVC solution; T = tests, in dilute homogenate. Bars represent standard deviations.

dra which can have behavioral effects. The effective concentration of metabolites, surrounding a hydra, can not be extrapolated from our data.

Two responses are elicited when homogenates are applied to the medium sur-

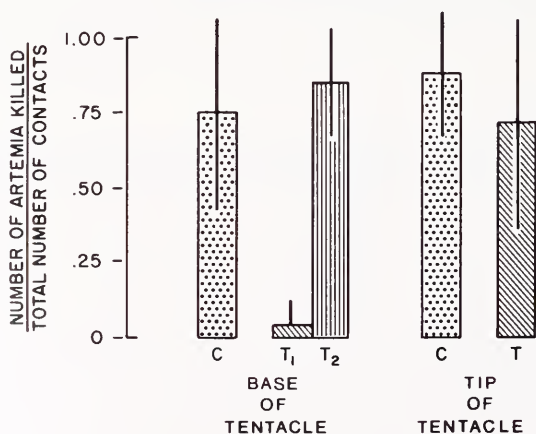


FIGURE 8. Bases and tips of intact tentacles were tested for inhibition of stenotele discharge in $1/25$ dilutions of homogenate. The base of the tentacle was covered with homogenate for 2 min and then the entire tentacle was tested with excess brine shrimp for 2 min. Inhibited regions of the tentacle were ablated, placed in fresh BVC solution, and retested after 1 h. The scale represents the ratio of the number of brine shrimp killed over the total number of contacts (mean $\pm$ SD). C = controls, tentacle placed in BVC solution only; T₁ = test, base of tentacle placed in homogenate; T₂ = retest, base of tentacle after washing for 1 h in BVC solution. $n = 8$.

rounding a hydra: (1) an activation of the feeding reflex (mouth opening and tentacle movement) and (2) an inhibition of stenotele discharge. The strong activation of the feeding reflex does not cause the decrease in the killing of brine shrimp because there is no decrease in killing when GSH is applied to the external medium (GSH causes a strong activation of the feeding reflex).

Ruch and Cook (1984) proposed that inhibition of stenotele discharge was due to the previous discharge of nematocysts. They found that instead of inhibition, brine shrimp homogenates (5.8 $\mu\text{g}/1.5\text{ ml}$) caused a 35% increase in stenotele discharge. Only when they applied a "crude homogenate" (of an unknown concentration) were they able to get a significant decrease in stenotele discharge. They attributed this inhibition to the large number of stenotele nematocysts that were discharged by the homogenate itself. According to their data 75 to 100 nematocysts were discharged per hydra.

We are unable to confirm these findings with our experiments. In our experiments, homogenates diluted to a similar protein concentration (1.8 $\mu\text{g}/\text{ml}$) induced 50% inhibition of stenotele discharge. The inhibition of stenotele discharge is not due to previous nematocyst discharge, since at higher protein concentrations (0.74 mg/ml), where there is complete inhibition of stenotele discharge, no significant amount of nematocyst discharge occurred. Our work is consistent with that of Pantin (1942) and Ewer (1947) who found that food extract alone does not elicit nematocyst discharge, although it does lower the threshold to discharge when applied locally. Since homogenate is the only variable altered in our experimental design, we believe that a substance in the homogenate causes the inhibition of stenotele nematocysts.

Ruch and Cook (1984) showed that nematocyst-rich tissues will cause complete inhibition of stenotele discharge. Furthermore, Clark and Cook (1986), in the colonial hydroid *Halocordyle disticha*, were able to induce complete inhibition using the discharge products of large numbers of purified stenotele nematocysts. It is possible that more than one mechanism for inhibition of stenotele discharge exists or that the various mechanisms may employ a similar substrate.

Gel filtration chromatography demonstrates that the substances activating the feeding reflex and those inhibiting stenotele discharge do not have the same molecular weight. The largest molecular weight fraction (>5000), which caused the strongest inhibition of stenotele discharge, displayed very little or no activation of the feeding reflex, whereas the samples causing the strongest feeding reflex also produced the highest level of killing. Since gel filtration chromatography separates substances based upon molecular weight, the partial inhibition in later column samples indicates either that inhibition of stenotele discharge may be caused by more than one substance or that there was incomplete separation of the homogenate samples.

Injections of brine shrimp homogenates into the basal disc of hydra do not cause any inhibition of stenotele discharge or activation of the feeding response until homogenate leaks into the external environment. Furthermore, different body regions or even different regions of a tentacle behave independently from each other. Whether inhibition occurs directly at the nematocyte or involves some other component of the battery cell complex (Hufnagel *et al.*, 1985) remains to be determined.

In summary, inhibition of stenotele discharge can be induced with external application of prey homogenates. The onset of inhibition is rapid ($<30\text{ s}$), while the release from inhibition is relatively slow ($>20\text{ min}$). Inhibition is dependent upon the concentration of homogenate. The inhibitory substances are different from those which activate the feeding reflex. The receptors for these inhibitory substances are found on the external surface of the hydra tentacle and may be associated with the nematocyte directly or with some other component of the battery cell complex.

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THE EFFECTS OF SALINITY STRESS ON THE RATES OF AEROBIC RESPIRATION AND PHOTOSYNTHESIS IN THE HERMATYPIC CORAL *SIDERASTREA SIDEREA*

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ABSTRACT

Corals are reputed to have low tolerance to salinity fluctuations. Yet the scleractinian coral *Siderastrea siderea* commonly inhabits reef zones and nearshore areas that experience salinity fluctuations of 5 to 10‰. Small colonies of this species were subjected to both long-term and sudden decreases or increases in salinity. Their rates of aerobic respiration and photosynthesis, measured as changes in oxygen concentration, were followed for up to 144 hours after the sudden changes.

Normal salinities of coastal waters near Panacea, Florida, are 28 to 30‰, but *S. siderea* was able to acclimate to 42‰ when salinity was increased slowly over a 30-day period. Neither respiratory nor photosynthetic rates of *S. siderea* were affected by changes in salinity of less than 10‰ above or below the acclimation salinity. Greater changes in salinity (either up or down) caused decreases in respiratory and photosynthetic rates proportional to the magnitude of the salinity change. Decreases in chlorophyll per algal cell and in assimilation number were associated with and possibly responsible for some of the decreases in photosynthetic rates.

These results show that *S. siderea* is able to withstand sudden and prolonged, environmentally realistic changes in salinity without measurable whole-animal effects. Further studies are needed to determine whether this species is remarkable in its ability to tolerate salinity change, or whether reef corals are more tolerant to salinity change than is generally believed.

INTRODUCTION

Observations on the distribution and vigor of coral reefs have suggested a relationship between major environmental factors and the physiological well being of hermatypic corals. In general, coral reefs only flourish within narrow ranges of salinity, temperature, and turbidity (Wells, 1957; Yonge, 1963; Stoddart, 1969; Connell, 1973; Glynn, 1973). Although there have been a few experimental studies on the effects of temperature and sedimentation on the physiology of corals, the effects of salinity remain poorly studied.

Support for the generally accepted relationship between salinity and coral reef distribution is indirect rather than experimental. Freshwater runoff or heavy rain on shallow reef flats, or coincident with low spring tides, may lower local salinities and lead to physiological damage to reef organisms. Excessive rain killed shallow water biota on reefs in Tahiti (Crossland, 1928) and Jamaica (Goodbody, 1961). Runoff following such storms may especially damage reefs close to river mouths (Squires,

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1962). The outflow of freshwater from a freshwater lens along atoll margins has been suggested to limit coral growth on inner reef flats, as observed on Arno atoll (Hiatt, 1957).

At the other extreme, high salinities resulting from prolonged drought have been observed to occur in the lagoons of Turneffe Atoll, Belize (Smith, 1941). Evaporation in tide pools during low tides may also cause salinities to rise to stressful levels.

It is generally agreed that most scleractinian corals can survive only small variations in salinity, with death resulting when salinity drops below 25‰ (Edmondson, 1928) or increases above 40‰ (Jokiel *et al.*, 1974). The few experimental studies of the effects of salinity on coral mortality have reported tolerances ranging from lower limits of 17.5 to 28‰ (Vaughan, 1919; Edmondson, 1928) and higher limits of 38.5 to 52.5‰ (Wells, 1957; Edmondson, 1928). Kinsman (1964), however, found reefs growing in salinities of more than 42‰ in the Persian Gulf with large heads of *Porites* spp. flourishing at 48‰. Observations of coral reef distribution will provide by inference information on the salinity tolerances of corals, however, there is a need for systematic studies on the effects of salinity on corals.

Detrimental effects of salinity on hermatypic corals can occur due to physiological stress on the coral animal or the corals' algal symbionts. The photosynthetic products of symbiotic zooxanthellae contribute to coral metabolism (Muscantine, 1967; von Holt and von Holt, 1968). If the symbiotic relationship between coral and algae is disrupted due to salinity stress, there may be a profound effect on coral metabolism. Several studies have reported the effects of temperature and light on zooxanthellae photosynthesis (Clausen and Roth, 1975; Coles and Jokiel, 1977; Jacques and Pilson, 1980; Jacques *et al.*, 1983) but similar information on the effects of salinity is lacking. Goreau (1964) observed that flood waters during Hurricane Flora lowered the salinity to less than 30‰ for more than 5 weeks resulting in mass loss of zooxanthellae by many reef flat corals.

The objective of this study was to determine the effects of changes in salinity on coral respiration and photosynthesis. The experimental organism was the hermatypic coral *Siderastrea siderea*, which has a wider geographic distribution than most west Atlantic reef corals. It has been reported as far north as North Carolina (MacIntyre and Pilkey, 1969) and is common wherever there is shallow hard substrate in the northeastern Gulf of Mexico. It is abundant on Floridian and Caribbean coral reefs, especially in shallow reef-flat and back-reef areas where salinity fluctuations are the greatest (A.M.S., unpub. obs.). In the Florida panhandle area of the Gulf of Mexico, this species occurs as small nodules 5 to 10 cm in diameter on rocky bottoms near shore. Salinities in these nearshore areas are usually 28 to 30‰ but can drop rapidly 5 to 10‰ during periods of runoff (unpub. obs.).

MATERIALS AND METHODS

Colonies of *Siderastrea siderea* 4 to 8 cm in diameter were collected from hard-bottom outcrops located 30 to 40 meters offshore of St. Teresa Beach, Florida, at a depth of 2 to 3 meters. They were maintained in a recirculating filtered seawater system in a constant temperature room. The volume of the system was approximately 400 liters of seawater. Water was changed each time a new batch of corals was collected, so that the initial salinity was identical to that at the time of collection. Lighting of approximately $100 \mu\text{Ein m}^{-2} \text{s}^{-1}$, measured with a Licor quantum sensor, was provided by banks of four 40 watt cool-white fluorescent bulbs suspended above the aquaria. Timers controlled a 12-hour light:dark cycle.

All salinity experiments were conducted at temperatures between 22 and 26°C.

During the winter months when the field temperatures were lower, the corals were brought to laboratory temperature by slowly raising the temperature a few degrees each day. Respiration and photosynthesis measurements were made daily until the laboratory temperature was reached. After one to four days at constant temperature, the salinity change was initiated. Tests with salinity changes of 30 to 25‰, 30 to 20‰ and 30 to 16‰ were done this way. The remaining tests were conducted during the summer at the temperature at which the corals were collected.

Each salinity test was begun by measuring the rates of aerobic respiration (oxygen consumption) and photosynthesis (oxygen production) for each coral at the control salinity (salinity at time of collection). Eight to ten corals and two control chambers without corals were used in each test. Measurements were made daily for two to four days before exposing some of the corals to the new salinity. Half of the corals ($n = 4$ or 5) were then exposed to the test salinity. A second set of incubations were begun immediately after, with the experimental group in the test salinity and the control group remaining at the environmental salinity. Thus, the first measurement of change in respiration was measured over the first hour after exposure to a new salinity, and photosynthesis over the second hour of exposure. The corals were later returned to maintenance aquaria at their respective salinities. Incubations were repeated at 24-hour intervals for up to a week.

During incubations, daytime respiration measurements were done first by covering the incubation system with several layers of black plastic sheeting to exclude light. A second incubation in the light to measure photosynthesis by the zooxanthellae was begun immediately following the dark incubation. Individual colonies were incubated in plexiglas chambers in a water bath to maintain constant temperature. Incubations lasted from 45 to 60 minutes. The dissolved oxygen concentration (DO) of the filtered seawater used to fill the chambers was measured and the seawater bubbled with air if it was less than 95% saturation. Water samples were taken from chambers at the beginning and at the end of the incubation. DO measurements were made using the Winkler method (Strickland and Parsons, 1972). Gross photosynthetic rates (hereafter referred to as photosynthetic rates) were estimated by summing oxygen production measured in the light with oxygen consumption measured in darkness.

At the end of each experiment, coral tissues were removed from the skeletons with a jet of filtered seawater from a Water-Pik (Johannes and Wiebe, 1970) and homogenized. Aliquots of the homogenates were preserved with Lugol's iodine (Thronsdon, 1978) for microscopic determination of zooxanthellae density, or filtered onto glass fiber filters for chlorophyll measurements using a fluorometric technique (Strickland and Parsons, 1972). Surface areas of each coral were measured by the aluminum foil method of Marsh (1970), and were used to normalize the oxygen flux rates of the various sized colonies (McCloskey *et al.*, 1978). Oxygen flux rates are reported as the mean ($n = 4$ or 5) ± 1 standard deviation. Statistical comparisons between means for each treatment were done using a two-tailed *t*-test.

RESULTS

Daytime respiration

All of the respiratory rates reported below for the salinity tests were measured during normal daylight hours. Before accepting this experimental protocol, we tested to determine whether respiratory rates measured thus were similar to respiratory rates measured during normal nighttime hours. The results (Table I) show that respiratory rates of individual colonies varied by as much as a factor of two. However, for each

TABLE I

Daytime versus nighttime respiratory rates of individual colonies of Siderastrea siderea

Colony number	Daytime respiration rate (nmol O ₂ cm ⁻² h ⁻¹)	Nighttime respiration rate (nmol O ₂ cm ⁻² h ⁻¹)
1	411	411
2	824	828
3	573	552
4	810	755
—	—	—
Mean	655 ± 207	637 ± 256

Each rate represents a single one-hour long measurement.

colony, respiratory measurements made during the daytime were similar to those measured at night. Therefore we accepted the procedure of measuring respiration rates during daytime incubations.

Temperature acclimation

As salinity tests were conducted year-round at similar temperatures (22–26°C), corals used in experiments conducted during the winter were necessarily exposed to large temperature increases just before exposure to salinity changes.

Respiration rates (R) of corals collected at 16°C ($R = 371 \pm 67$ nmol O₂ cm⁻² h⁻¹; $n = 10$) increased significantly when water temperatures were gradually increased to 22°C over a 72 hour period ($R = 532 \pm 90$ nmol cm⁻² h⁻¹; $P < 0.01$) (Fig. 1). Photosynthetic rates also increased significantly ($P < 0.005$) from 890 ± 200 nmol O₂ cm⁻² h⁻¹ at 16°C to 1462 ± 229 nmol O₂ cm⁻² h⁻¹ at 22°C during the same time period. Photosynthetic rates increased more (39%) than respiratory rates (30%). The Q_{10} s calculated according to Schmidt-Nielsen (1979) were 1.82 for respiration and 2.29 for photosynthesis.

Responses to sudden changes in salinity

Eight tests were conducted: six on the effect of decreases and two on the effects of increases in salinity on respiratory and photosynthetic rates. Corals in the first three tests (30‰ to 25, 20, and 16‰) were collected at 15°C and were brought to laboratory temperatures, as described above, before beginning the salinity changes. Two tests studied the responses of corals acclimated to 42‰ when exposed to salinities of 35 and 22‰.

The mean respiratory and photosynthetic rates of control and test groups before and at the end of the exposure periods are summarized in Table II. There was a large amount of variability between individual colonies before exposure, and mean rates of the control groups often increased or decreased during the exposure period. However, the mean rates of the controls were similar to those previously reported for reef corals (Wethey and Porter, 1976; McCloskey *et al.*, 1978; Davies, 1978; Szmant-Froelich *et al.*, 1981). The post-exposure values in Table II are the mean rates measured at the end of each salinity test, and tests varied in duration of exposure. However, whenever a significant effect was seen, it generally occurred within a few hours of exposure (Fig. 2).

Overall, the mean change in respiration of the control corals and of the corals

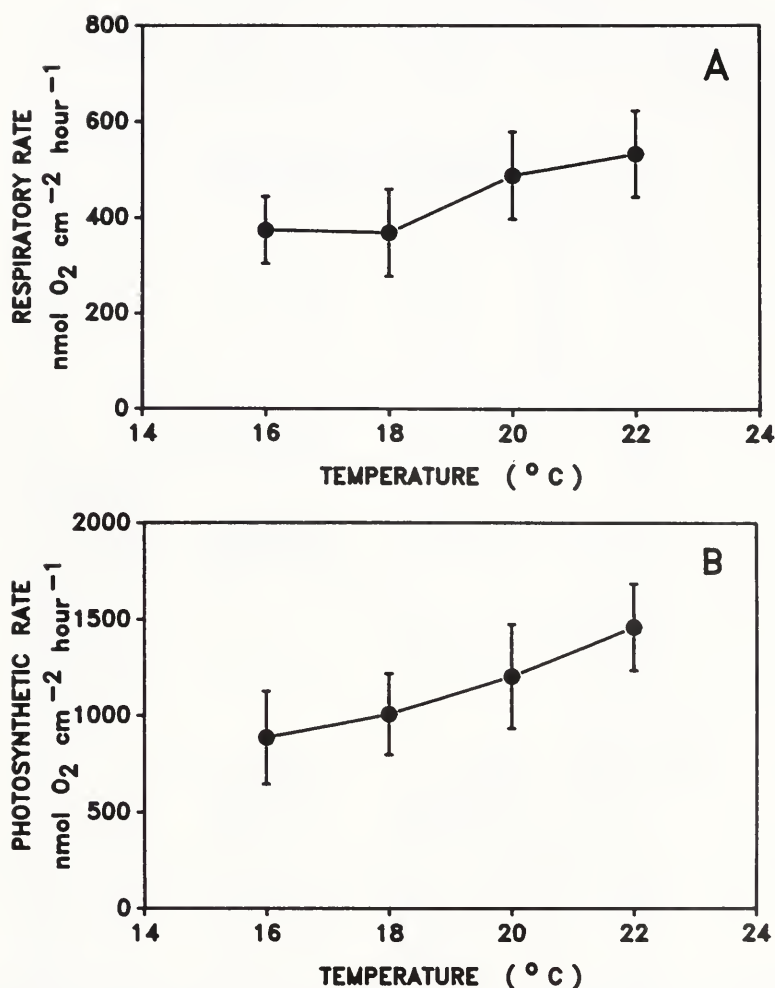


FIGURE 1. Mean (± 1 S.D.) rates of respiration (A) and gross photosynthesis (B) of colonies of *Siderastrea siderea* exposed to 2°C increases in temperature every 24 hours. $n = 10$.

exposed to salinity changes of 5‰ and 7‰ was an increase of $67 \pm 116 \text{ nmol O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ ($n = 40$), and this mean was not significantly different from zero ($t = 1.84$, $P > 0.05$). The mean change in photosynthesis for the same groups was an increase of $157 \pm 181 \text{ nmol O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ ($n = 40$), and this mean change was significantly greater than zero ($t = 2.48$, $P < 0.02$). The gradual increase over time in photosynthesis by the control corals could be explained as a gradual adaptation by their zooxanthellae to higher light levels in the laboratory as compared to the low light levels in the muddy coastal waters where the corals were collected. These changes (increases) in respiratory and photosynthetic rates exhibited by the control corals over the one to five day experimental periods (Table II) are, in general, small and in the opposite direction to the more dramatic changes (decreases) in respiratory and photosynthetic rates exhibited by test corals exposed to the larger changes in salinity. In two cases for respiration (42 to 35‰ tests; Table II) and in three cases for photosynthesis (same two plus 42 to

TABLE II

*Respiratory and photosynthetic rates of colonies of Siderastrea siderea before and after being subjected to sudden changes in salinity for up to 144 hours. Student's *t*-test was used to test for the significance of differences between control and experimental groups of corals at the end of the exposure period. Values are means $\pm$ 1 S.D. Values of *n* in () can be found with the respiration results*

Salinity change	Duration of exposure (hours)	Respiration (nmol O ₂ cm ⁻² h ⁻¹)				Photosynthesis (nmol O ₂ cm ⁻² h ⁻¹)					
		Control pre-exposure	Control at end of exposure period	Experimental pre-exposure	Experimental at end of exposure period	Control vs. experimental*	Control pre-exposure	Control at end of exposure period	Experimental pre-exposure	Experimental at end of exposure period	Control vs. experimental*
Decrease in salinity											
30 to 25‰	144	452 ± 180 (15)	260 ± 59 (5)	366 ± 98 (15)	231 ± 58 (5)	n.s.	1677 ± 831	1077 ± 109	1540 ± 597	1144 ± 279	n.s.
30 to 20‰	48	280 ± 69 (20)	257 ± 51 (5)	316 ± 74 (20)	257 ± 66 (5)	n.s.	890 ± 253	650 ± 175	1001 ± 296	797 ± 215	n.s.
30 to 16‰	24	382 ± 120 (15)	429 ± 225 (5)	428 ± 64 (15)	277 ± 17 (5)	<i>P</i> < .025	1056 ± 298	1462 ± 229	1008 ± 245	473 ± 67	<i>P</i> < .025
28 to 16‰ ¹	119	474 ± 52 (12)	442 ± 60 (4)	455 ± 79 (12)	286 ± 56 (5)	<i>P</i> < .025	1548 ± 182	1535 ± 121	1576 ± 355	709 ± 138	<i>P</i> < .025
42 to 35‰	94	547 ± 113 (16)	596 ± 149 (4)	419 ± 121 (16)	560 ± 46 (5)	n.s.*	1527 ± 234	1679 ± 333	1172 ± 303	1563 ± 505	n.s.*
42 to 22‰	17	626 ± 145 (12)	883 ± 43 (4)	577 ± 142 (12)	106 ± 92 (5)	<i>P</i> < .001	1812 ± 429	1896 ± 588	1352 ± 242	322 ± 173	<i>P</i> < .001*
Increase in salinity											
32 to 42‰	98	535 ± 90 (12)	536 ± 143 (4)	402 ± 70 (12)	397 ± 56 (4)	n.s.*	1421 ± 222	1807 ± 92	922 ± 112	691 ± 125	<i>P</i> < .001*
28 to 42‰	20	481 ± 56 (16)	498 ± 51 (4)	487 ± 83 (16)	390 ± 78 (4)	<i>P</i> < .025	1225 ± 188	1489 ± 156	1271 ± 320	915 ± 280	<i>P</i> < .025

¹ Salinity was further lowered to 12‰ over a 60 hour period before Chl_a and zooxanthellae counts were made.

* Control and experimental groups were significantly different before exposure to the change in salinity; statistical comparisons were made between pre- and post-exposure rates of the experimental group.

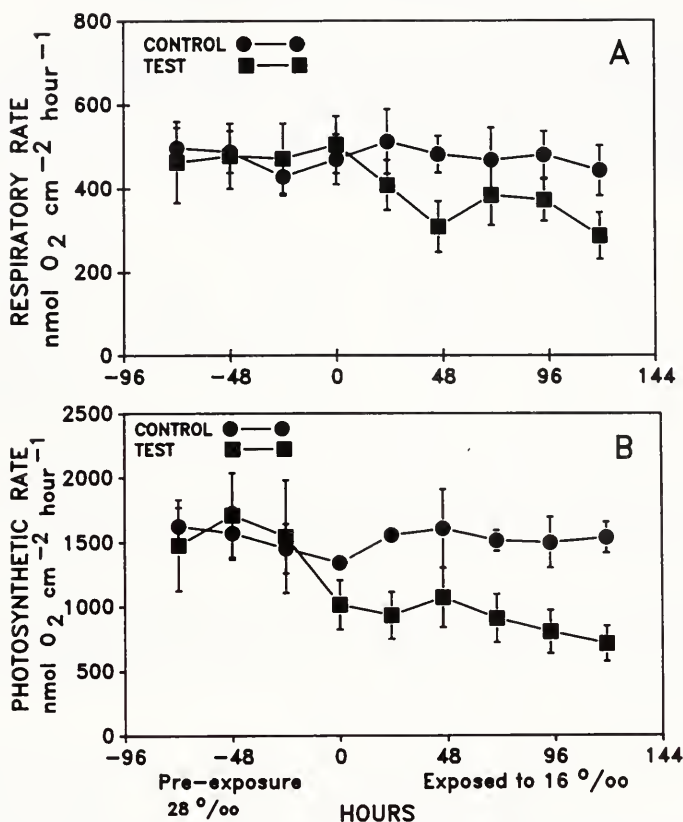


FIGURE 2. Mean (± 1 S.D.) respiration (A) and photosynthesis (B) rates of *Siderastrea siderea* acclimated to 28‰ salinity and abruptly exposed to 16‰ salinity for a period of one week. $n = 4$.

22‰; Table II) pre-exposure rates of the control groups were significantly different from those of the experimental groups. In these cases, post-exposure rates of the experimental groups were tested against their own pre-exposure rates.

Salinity decreases of up to 10‰ (e.g., 30‰–20‰) caused no significant effect on either respiratory or photosynthetic rates of test corals, but a decrease of 14‰ (from 30‰ to 16‰) caused a significant decrease in both rates (t -test, $P < 0.025$). Respiration decreased by 48% after 19 hours of exposure and photosynthesis decreased by 67% after 20 hours of exposure to 16‰. This particular salinity change was repeated during the summer when the ambient temperature at the collection site was the same as that in the laboratory; ambient salinity, however, was slightly lower (28‰). The corals in this later test group also showed a rapid and significant decrease in both respiration and photosynthesis when exposed to 16‰ ($P < 0.025$) (Fig. 2). Respiration decreased by 36% and photosynthesis by 33% after 44 hours of exposure, similar to the decreases observed in the first test. Further gradual lowering of salinity caused the rates of both respiration and photosynthesis to continue to decrease to 53% and 56% (respectively) of the original rates. The corals eventually died after exposure to 12‰.

Salinity increases from 32‰ to 42‰ ($\Delta 10$ ‰) caused no significant change in res-

TABLE III

Zooxanthellae density and chlorophyll content of the zooxanthellae of Siderastrea siderea colonies used in the salinity tests. Measurements were made shortly after the end of each experiment. Control corals are those that were maintained at normal salinities throughout the test period. Test corals are those that were exposed to lowered salinities for the durations listed in Table II.

Salinity change ‰	Zoox. density 10 ⁶ cells/cm ²		Chlorophyll <i>a</i> µg/cm ²		Chl <i>a</i> /Zoox. µg/10 ⁶ cells	
	Control	Test	Control	Test	Control	Test
30 to 25 (5)	1.49 ± 0.30	1.32 ± 0.20	9.6 ± 1.8	8.8 ± 3.0	6.6 ± 0.9	7.0 ± 2.7
30 to 20 (5)	1.46 ± 0.36	2.00 ± 0.34**	16.4 ± 4.9	14.0 ± 1.3	12.1 ± 5.5	7.1 ± 1.4
28 to 12* (4)	0.72 ± 0.11	0.53 ± 0.15	7.2 ± 1.6	3.4 ± 1.2**	10.3 ± 3.7	6.4 ± 1.8
42 to 35 (4)	0.96 ± 0.07	0.86 ± 0.07	9.1 ± 1.3	6.9 ± 1.1**	9.5 ± 2.0	8.1 ± 1.8
42 to 22 (4)	—	—	9.8 ± 1.7	6.7 ± 2.7	—	—
Mean	1.19 ± 0.41	—	10.8 ± 4.5	—	9.6 ± 3.9	—

* This is the same test that is listed in Table II as a change in salinity from 28 to 16‰. Salinity was further reduced to 12‰ after the conclusion of the test and before the tissues were collected for the zooxanthellae and chlorophyll measurements.

** Denotes test groups that were significantly different from their controls (*t*-test, *P* < 0.05). Values are means ± 1 S.D. (n).

piration, but did cause a 25% decrease in photosynthesis (Table II). Corals exposed to a 14‰ increase (from 28‰ to 42‰) had significant decreases in both respiration and photosynthesis (*P* < 0.025). Photosynthesis decreased by 39% after 47 hours and respiration decreased by 22% within 43 hours.

Several colonies of *S. siderea* were gradually acclimated to 42‰ by allowing the seawater in the recirculating system to slowly evaporate over a month-long period. Respiratory and photosynthetic rates of these acclimated colonies were similar to those collected at 28 to 30‰ (Table II). When these acclimated corals were exposed to a 7‰ decrease in salinity (to 35‰) there were no measurable effects, but there were large decreases in respiration and photosynthesis when corals acclimated to 42‰ were exposed to 22‰, a change in salinity of 20‰. Respiration decreased by 82% and photosynthesis by 81% after 12 hours of exposure.

Zooxanthellae density and chlorophyll content

The control corals had variable zooxanthellae densities ranging from 0.82 to 1.49 × 10⁶ cells/cm² (Table III) in measurements made after the completion of the salinity tests. There were no significant differences between the zooxanthellae densities of control and test groups, except for the 30 to 20‰ test group which had a significantly greater zooxanthellae density than its control. The chlorophyll content of the control corals ranged from 5.8 to 17.4 µg/cm². In all cases, the experimental corals had lower chlorophyll contents than their control groups, but the differences were only statistically significant (*t*-test, *P* < 0.05) for the 28 to 12‰, and 42 to 35‰ salinity tests (Table III). In the former tests the decrease in chl *a* coincided with significant reductions in photosynthetic rates.

DISCUSSION

Temperature effects

Oxygen consumption is the standard method for measuring routine metabolism of an animal and is generally equated to aerobic respiration (Prosser, 1973). Within

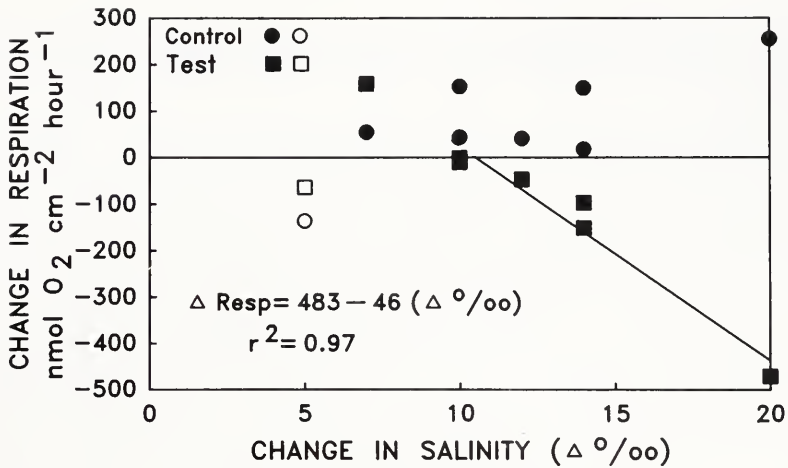


FIGURE 3. Plot of the mean change in respiration rate of colonies of *Siderastrea siderea* after 24 hours of exposure to reduced or increased salinity (Y axis) versus the change in salinity (X axis). Change in respiration rate was calculated for each coral as the difference between its respiration rate measured 24 hours after exposure and the mean of several measurements made over a 24 to 48 hour period before the exposure began. The changes in respiration of the control groups were plotted versus the changes in salinity of their respective test groups rather than opposite a zero change in salinity. The regression line was calculated using a least squares method; the 5‰ salinity change point (0) was omitted from the regression for reasons explained in the text. $n = 4$ or 5.

the temperature range that an organism can tolerate, the rate of oxygen consumption of heterothermic animals is often found to increase in a fairly regular manner with increasing temperature. The average Q_{10} over the temperature range of 16 to 22°C for the respiration rate of *Siderastrea siderea* was 1.82, which is similar to the Q_{10} s for respiration reported for various coelenterates (Lenhoff and Loomis, 1957; Sassman and Mangum, 1970; Mangum *et al.*, 1972). Photosynthesis is also influenced by temperature in a similar manner. The average Q_{10} for photosynthesis by the zooxanthellae of *S. siderea* was 2.29 for the same temperature range. This compares well with those found for many plants (1.0–2.7) (Salisbury and Ross, 1969).

Responses to changes in salinity

According to Vernberg and Vernberg (1972), there are several typical physiological responses to salinity stress. These include (a) an increase in respiration when subjected to salinity stress (regardless of the direction of salinity change); (b) a decrease in respiration regardless of direction of salinity change; (c) an increase in respiration if salinity is lowered but a decrease if salinity is increased; and (d) no change in respiration. In addition, there is often an initial transitory change in respiration after which a new steady state is achieved.

Siderastrea siderea shows a combination of responses (b) and (d) above, depending on the magnitude of the salinity change (Fig. 3, 4). Changes in salinity of less than 10‰ had no significant effect on respiration, nor, in many cases, on photosynthesis (response d). Thus, within the environmentally realistic salinity range for this species *S. siderea* is able to tolerate sudden and prolonged exposures to fairly large (10‰) changes in salinity. Changes in salinity greater than 10‰ and 9‰ caused significant decreases in respiration and photosynthesis, respectively, regardless of whether salin-

ity was increased or decreased (response b). There was no initial transitory burst of respiratory activity within the first hour of exposure to the altered salinity (results in Fig. 2 are representative). Rates remained lower for at least a week during the present experiments.

Figure 3 includes a least-squares linear regression of the change in respiration after 24 hours of exposure to altered salinity *versus* the change in salinity over that period. The data from the $\Delta 5\%$ test were omitted from the regression because we suspect these animals of having been incompletely adjusted to the laboratory temperature at the time the salinity change test began, and simultaneous changes in salinity and temperature may have a synergistic detrimental effect (Coles and Jokiel, 1978). All control groups (except for the $\Delta 5\%$ one) and the $\Delta 7\%$ experimental group had increases in respiration over the 24-hour experimental period. The regression shows that only salinity changes greater than 10.5% resulted in respiration depression. If this coral is an osmoconformer like many coelenterates (Ranklin and Davenport, 1981), its extracellular fluid osmotic pressure will fall or rise with changes in the environment. This would lead eventually to cellular swelling or shrinking, and to cell disruption. But even before such damage is done, the changes in cell size and alterations in internal geometry might disturb cell functions and possibly cause decreases in metabolic rates (Ranklin and Davenport, 1981). Alternatively, corals could contract their polyps thus reducing their contact with the adverse salinity conditions. Shumway (1978) has shown that the sea anemone *Metridium senile* contracts when exposed to lower salinity. Contraction would (a) decrease gas exchange with the exterior, and thus contribute to lower respiration rates, and (b) reduce the exposure of the zooxanthellae to light, and contribute to a lower rate of photosynthesis. However, contraction might have a similar effect on oxygen flux rates regardless of the magnitude of the change in salinity. Thus it is not a completely satisfactory explanation for the linear decrease in respiratory and photosynthetic rates that occurred as the size of the salinity change increased. No systematic observations on changes in coral behavior due to salinity change were carried out. However, casual observations revealed that *S. siderea* exposed to high or low salinities sometimes retracted into their skeletons for extended periods. Further studies should include experiments on the effectiveness of retraction as an avoidance mechanism in corals, and on the effect of retraction on the measurement of respiratory and photosynthetic rates.

The ability of this species to acclimate to abnormally high salinities (42%) shows that the animal can acclimate to a large net salinity change when the change occurs slowly, whereas the same salinity change if experienced suddenly might be fatal. As recovery experiments were not performed, we do not know at what point the depression in respiration is still reversible upon return to normal salinities, nor whether acclimation after a sudden large salinity change will occur. In the single longer experiment where salinity effects were found early on (Table II: 28 to 16%), respiration was still depressed after 5 days of exposure to the lower salinity.

Figure 4 shows a similar linear regression of change in photosynthesis after 24 hours of altered salinity *versus* change in salinity. The minimum salinity change that caused depression in photosynthesis was 9% which is slightly lower than that required to cause a depression in respiration. There were also large decreases in the chlorophyll content per algal cell in all of the test groups where it was measured, except for the 30 to 25% salinity change group (Table III). The decreases in photosynthesis appear to have been caused by the combined effect of small decreases in zooxanthellae density, chlorophyll content per algal cell and, in one case, by a decrease in the assimilation number (oxygen produced per amount of chlorophyll, Table IV). Polyp retraction also could have played a role as discussed above.

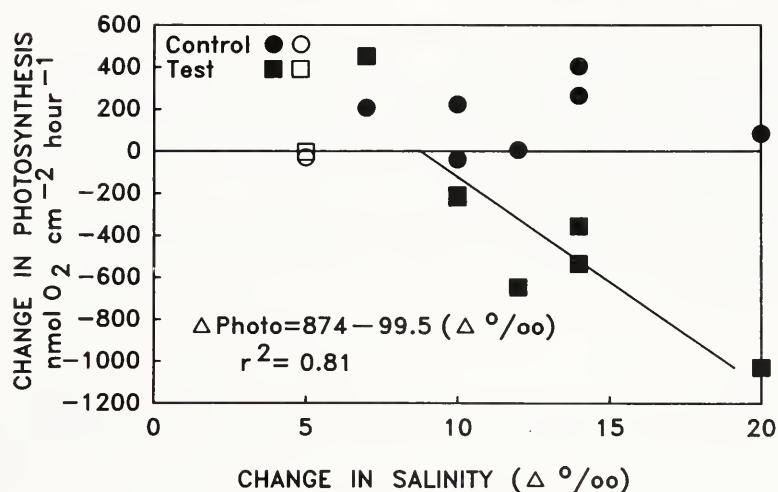


FIGURE 4. Plot of the mean change in photosynthesis rate of colonies of *Siderastrea siderea* after 24 hours of exposure to altered salinity (Y axis) versus the change in salinity (X axis). See legend of Figure 3 for further explanation. $n = 4$ or 5.

In conclusion, respiratory and photosynthetic rates of the coral *Siderastrea siderea* were not adversely affected by changes in salinity of less than 10‰, although their chlorophyll content began to decline after changes of 7‰ salinity. When exposed to salinity changes greater than 10‰ for 24 hours, respiratory and photosynthetic rates both decreased regardless of whether salinity was increased or decreased. Linear regressions showed a significant linear relationship between the degree of respiratory and photosynthetic depression and the magnitude of the salinity change. Finally, it is important to note that scleractinian corals are generally considered intolerant to salinity change, yet *S. siderea* was able to experience rather large (10‰) changes in salinity without any demonstrable effect on respiratory or photosynthetic rates. It will be interesting to determine whether this species is remarkable in this regard, and thus suited to live in reef-flat and coastal areas where salinity changes are more frequent, or whether scleractinian corals are more tolerant to salinity change than is generally believed.

TABLE IV

Photosynthetic performance by the zooxanthellae of *Siderastrea siderea* exposed to changes in salinity. Duration of exposure for each test can be found in Table II

Salinity Change ‰	nmol O ₂ (10 ⁶ Cells) ⁻¹ h ⁻¹		nmol O ₂ (μg chl a) ⁻¹ h ⁻¹	
	Control	Test	Control	Test
30 to 25 (5)	775 ± 135	825 ± 95	120 ± 25	138 ± 59
30 to 20 (5)	453 ± 118	393 ± 85	44 ± 19	57 ± 15
28 to 12 (4)	2173 ± 342	1350 ± 614**	222 ± 42	228 ± 133
42 to 35 (4)	1797 ± 448	1968 ± 566	188 ± 21	260 ± 121
42 to 22 (4)	—	—	192 ± 52	56 ± 35**

** Denotes test groups that were significantly different from their controls (t -test, $P < 0.05$). Values are means ± 1 S.D. (n).

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UPSTREAM AND DOWNSTREAM CAPTURE DURING SUSPENSION FEEDING BY *OLIGOMETRA SERRIPINNA* (ECHINODERMATA: CRINOIDEA) UNDER SURGE CONDITIONS

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ABSTRACT

The crinoid *Oligometra serripinna* is a suspension feeder that usually experiences unidirectional tidal currents from which it extracts food particles by downstream capture (*i.e.*, while the food grooves face downcurrent). However, near slack tide, wave surge may cause brief current reversals, each lasting about 2 s at roughly 10 s intervals. To test if a crinoid can engage in upstream capture (*i.e.*, while the food grooves face upcurrent) during brief current reversals, we approximated these surge conditions in a laboratory flume. In the laboratory, as in the field, the crinoid oriented its food grooves downstream with respect to the predominant current, and the body posture did not change during the brief intervals of reversed flow. Brine shrimp cysts were added to the flume, and video recordings were made of the crinoid capturing these particles. Under surge conditions, the crinoid (1) captured 16.2% of the approaching particles while its food grooves faced downstream and (2) captured 8.0% of the approaching particles while its food grooves faced upstream. Thus *O. serripinna* used its filter both for upstream capture and for downstream capture, although the former was only about half as efficient as the latter.

INTRODUCTION

Many suspension feeding animals use a portion of their body as a filter to capture particles from the passing water. The filter parts that capture and transport the particles may be oriented toward the current (*i.e.*, upstream capture, as in bivalves) or away from the current (*i.e.*, downstream capture, as in entoprocts). Although these definitions work well for many filter feeders, there is some question as to whether crinoids, which are generally considered downstream capturers (Magnus, 1967; Meyer, 1979, 1982; Holland *et al.*, 1986), might sometimes function as upstream capturers. In some crinoids, an animal may orient some parts of its filter upstream and other parts downstream (*e.g.*, in the cone posture and in the partial arm fan described by La Touche, 1978, and by Byrne and Fontaine, 1981). It has been implied but not clearly shown that such crinoids can engage in upstream and downstream capture simultaneously. Furthermore, in another crinoid (*Oligometra serripinna*), the filter is oriented downstream in the unidirectional tidal current that predominates in the field. However, at slack tide, wave surge causes periodic reversals in the current direction (Leonard *et al.*, 1987). During each reversal, *O. serripinna* does not change its body posture. Thus the filter is oriented upstream. The present note (1) demon-

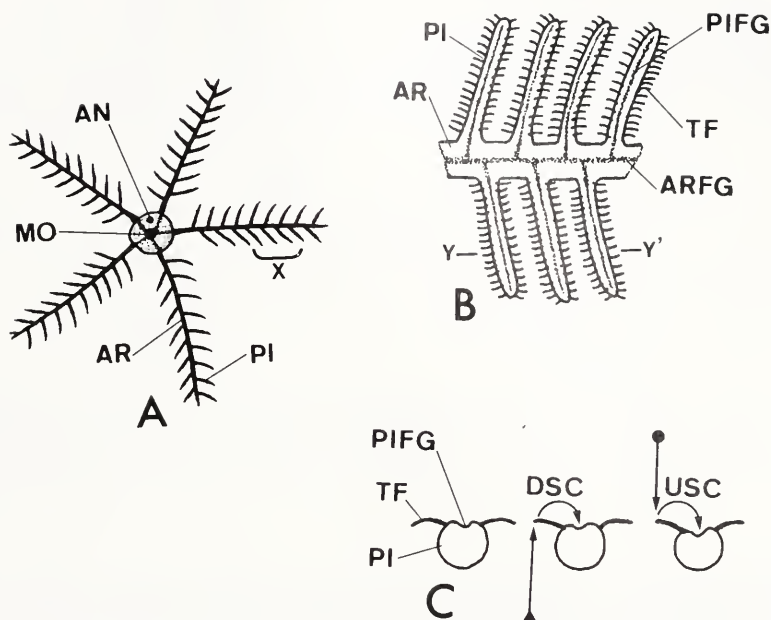


FIGURE 1. (A) Diagram of a crinoid showing the oral (mouth-bearing) side of the body. The mouth and anus open on the central disc from which the arms radiate. Each arm (usually more numerous than the 5 shown here) is fringed on either side by short side branches called pinnules. (B) Enlargement of the region opposite bracket x in Figure 1A. The arm food groove and pinnular food grooves run along the oral side of the arm and pinnules, respectively. Each pinnule is fringed by tube feet. (C) Cross section of three adjacent pinnules cut through plane y-y' in Figure 1B. The pinnular food grooves are on the oral side of the body, which is depicted facing upward. Downstream capture is diagrammed for the triangular particle, and upstream capture is diagrammed for the circular particle. Abbreviations: AN = anus, AR = arm, ARFG = arm food groove, TF = tube foot, USC = upstream capture.

strates that under surge conditions *O. serripinna* can alternate between upstream and downstream capture and (2) compares the efficiencies of these two processes.

The crinoid filter is diagrammed in Figure 1A,B. Approaching particles adhere to the adhesive tube feet, which rapidly bend and transfer the particles into the food grooves for transport to the mouth. The gut openings and food grooves are situated on the oral side of the body (*i.e.*, the side on which the mouth opens), which faces downcurrent during downstream capture. This behavior has been studied in detail for *O. serripinna* by Holland *et al.* (1986) and by Leonard *et al.* (1987). Downstream capture and upstream capture are shown diagrammatically in Figure 1C.

MATERIALS AND METHODS

We studied a feather star, *Oligometra serripinna*, living on sea fans at a depth of 10 m at North Reef, Lizard Island (14°38'S; 145°28'E), Great Barrier Reef, Australia. Current speed and direction in the microhabitat of *O. serripinna* were determined from underwater video recordings of a dye trail released by the non-motorized dye injector described by Colman *et al.* (1984). The study site and orientation of the injector relative to the crinoid are described by Leonard *et al.* (1987). We analyzed

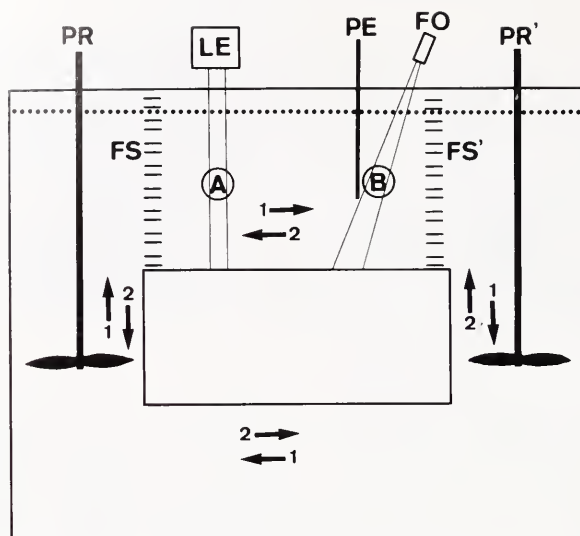


FIGURE 2. Diagram of 11-liter flume (45 cm long) for producing surging flow; the water level is indicated by the dotted line. To obtain flow in the predominant direction (indicated by arrows labeled 1), one propeller (=PR) is turned clockwise and the other (=PR') is turned counterclockwise for 6 s. To obtain flow in the reverse direction (indicated by arrows labeled 2), the action of each propeller is reversed for 2 s. At either end of the working section of the flume, there is a flow straightener (=FS and FS'). A bright beam of light is focused into the flume from above with a lens (=LE). A first video system records the speed and direction of brine shrimp cysts visualized in side view at the position marked by the circled A. An open mesh perch (shown in side view, =PE) supports the crinoid (not illustrated), which is illuminated with fiber optics (=FO). A second video system records the upstream and downstream captures in side view at the position marked by the circled B.

100 s of a recording made near slack tide at about 8:15 h on 16 August 1986. During every third of a second (*i.e.*, over 10 consecutive video frames), the speed and direction of the current was measured from the movement of irregularities in the dye stream. The predominant current toward the east-northeast was recorded as positive, and reversed current toward the west-southwest was recorded as negative.

The surge in the field was approximated in the laboratory in an 11-liter flume (Leonard *et al.*, 1987) to which an additional propeller and flow straightener had been added (Fig. 2). The two propellers were turned by a reversible, adjustable speed motor. One propeller turned clockwise and the other turned counterclockwise for 6 s to produce a *predominant flow* of approximately 5 cm/s. The action of the two propellers was then quickly reversed to produce a *reverse flow* for 2 s before the predominant flow was re-established and the cycle repeated.

The surge experiment in the flume was performed at 25°C, the approximate temperature in the field at the time. A specimen of *Oligometra serripinna* was allowed to attach to an open mesh perch with the food grooves facing downstream relative to the predominant current direction. Brine shrimp cysts that had been soaked in seawater for 8 h were then added to the flume. The concentration and speed of the cysts (and thereby the current speed) were measured as they passed through a segment of a light beam 2 cm in diameter and 1.8 cm from top to bottom (at position A in Fig. 2). The passing cysts were recorded in side view with a video system (Leonard *et al.*, 1987). A 100-s interval on the tape was quantified. Current speed was determined from the distance travelled by cysts over three consecutive video frames (*i.e.*, over an

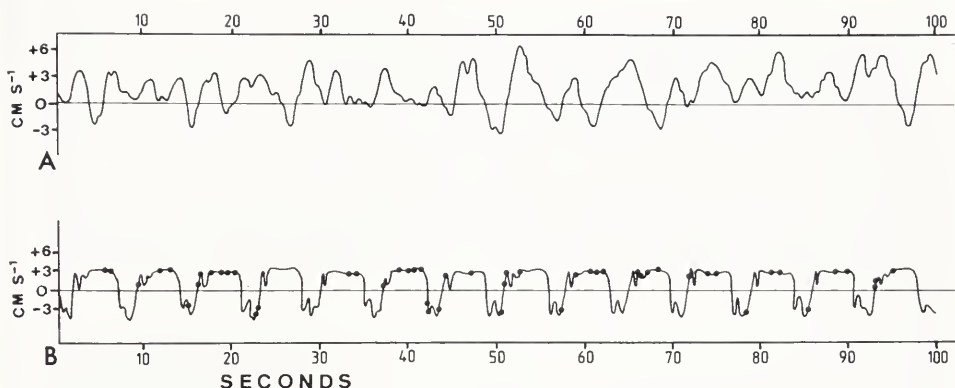


FIGURE 3. (A) Fluctuations in current velocity and direction (positive values are toward the east-northeast, and negative values are toward the west-southwest) in the microhabitat of *Oligometra serripinna*. (B) Fluctuations in current velocity and direction in a laboratory flume with surging flow (positive values are in the predominant direction, and negative values are for the relatively brief intervals of reversed flow). Each filled circle marks a point in the flow cycle where a brine shrimp cyst was captured.

interval of 0.1 s). Motion in the predominant direction was recorded as positive, and motion in the reverse direction was recorded as negative. During the same 100-s interval, one third of a single *O. serripinna* arm, with 20 pinnules (10 on each side), was recorded with a second video system, a duplicate of the first. The arm was recorded (at position B in Fig. 2) in side view a few degrees off the perpendicular to provide a foreshortened view of the pinnules on either side.

RESULTS AND DISCUSSION

Figure 3A plots fluctuations in current speed and direction near slack tide in the microhabitat of *Oligometra serripinna*. During the 100-s period, there were 10 conspicuous reversals in current direction, most "negative flow" periods lasted about 2 s and reached speeds of approximately 3 cm/s .

The results of the laboratory flume surge experiment are shown in Figure 3B, which places cyst capture in the context of fluctuations in current speed and direction. During the 100-s period, 41 cysts were captured during the cumulative 70 s that flow was in the predominant direction (*i.e.*, downstream captures), and 10 cysts were captured during the cumulative 30 s that flow was in the reverse direction (*i.e.*, upstream captures). The video recordings showed that all the food grooves faced directly upstream throughout each current reversal. Thus, upstream capture was unequivocal.

The capture efficiencies during downstream and upstream capture, respectively, were calculated by counting the cysts crossing the light beam during the 70 s of predominant flow and during the 30 s of reverse flow. Each count was divided by the recorded area of the light beam normal to the flow (3.8 cm^2) and multiplied by the recorded area of the arm normal to the flow (1.2 cm^2). This gave the number of cysts approaching the recorded part of the crinoid's filter as 252 and 125 during predominant and reversed current flow, respectively. The capture efficiency (percentage of captures normalized to approaches) was 16.2% during downstream feeding and 8.0% during upstream feeding. We do not know why upstream capture was markedly less efficient than downstream capture. An explanation of this difference would probably

require a detailed knowledge of flow through the gaps in the filter and the thickness of the boundary layers around the tube feet and pinnules.

The distinction between upstream capturers and downstream capturers appears to be useful for suspension feeders that actively pump water past their filters in a single direction. However, this distinction may not apply for passive suspension feeders, which depend on exogenous currents to bring particles to their filters. For example, Patterson's (1984) work indicates that a given polyp of an octocoral can engage in either upstream or downstream capture depending on the strength and direction of the exogenous current. Moreover, the present study is the first clear demonstration that at least some crinoids can augment their usual downstream capture with upstream capture.

In the field, *Oligometra serripinna* probably obtains only a small proportion of its food by upstream capture because there are no flow reversals during most of the tidal cycle (Leonard *et al.*, 1987) and upstream capture is markedly less efficient than downstream capture. Even so, it is possible that upstream capture may be relatively important in some other crinoid species—especially those living within the infrastructure of reefs where slow, meandering flows predominate.

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EELGRASS WASTING DISEASE: CAUSE AND RECURRENCE OF A MARINE EPIDEMIC

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ABSTRACT

Eelgrass populations are currently infected with a disease that produces symptoms and epidemiology reminiscent of the famous eelgrass wasting disease of the 1930s. This disease virtually eliminated eelgrass (*Zostera marina* L.) in the North Atlantic for three decades. For 50 years scientists have speculated about the cause of the 1930s eelgrass decline. We have now proven that the causal organism of the present epidemic is a pathogenic strain of *Labyrinthula*, which was suspected, but never conclusively shown to cause the 1930s wasting disease. We have isolated the infectious form of *Labyrinthula* from eelgrass from Maine to North Carolina on the Atlantic coast, and from Puget Sound on the Pacific coast; disease-related dieoffs of eelgrass beds are confirmed in Maine, New Hampshire, and Massachusetts.

DISCUSSION

A recurrence of the wasting disease, which almost eliminated eelgrass (*Zostera marina* L.) in the 1930s, was discovered on the border of New Hampshire and Maine in the early 1980s (Short *et al.*, 1986). Since then, eelgrass populations exhibiting symptoms and epidemiology comparable to the 1930s epidemic have been found from Nova Scotia to North Carolina. The eelgrass wasting disease of the 1930s constituted a marine epidemic which disrupted highly productive coastal ecosystems and fisheries. The disease had run its course by the 1940s; healthy eelgrass populations generally were reestablished by the 1960s. Over the past 50 years, scientists have proposed pathogenic organisms, temperature changes, reduced light, and combined environmental factors as causes of the 1930s disappearance of eelgrass. In this report, we present proof that the causal organism of the current epidemic is a pathogenic strain of *Labyrinthula* and describe our findings concerning the range of the disease.

The wasting disease that devastated eelgrass populations throughout the North Atlantic between 1930 and 1933 dramatically disrupted coastal and nearshore ecosystems. The most obvious impact was the reduction or loss of migratory waterfowl populations (Addy and Aylward, 1944). Equally important, though not immediately apparent, was the impact on commercial fisheries. The loss of the scallop fishery in the mid-Atlantic coast of the United States is best documented (Thayer *et al.*, 1984). The 1930s eelgrass loss altered coastal habitats and changed for decades the characteristics of nearshore soft sediment environments (Rasmussen, 1973, 1977). In fact, some locations were permanently altered, and eelgrass never returned.

The cause of the 1930s wasting disease was never conclusively determined (Johnson and Sparrow, 1961; Den Hartog, 1987). However, two main alternative theories

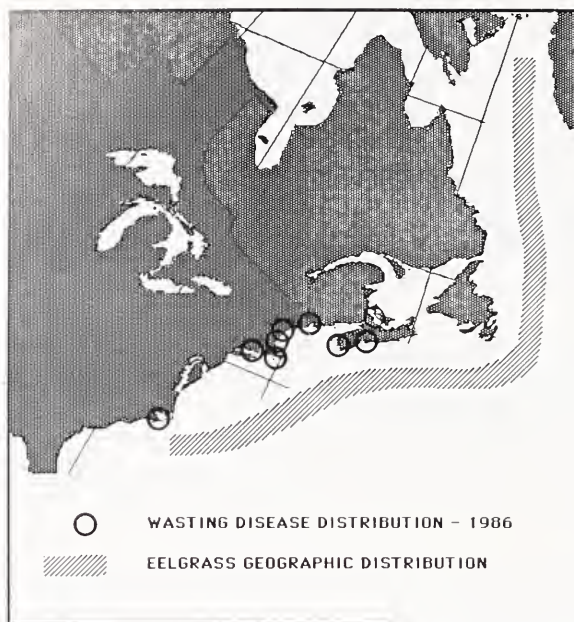


FIGURE 1. Geographic distribution of eelgrass and of the current wasting disease on the east coast of North America.

were promoted: first, that a microorganism was infecting and killing plants (Peterson, 1934; Renn, 1935), and second, that environmental stress from abnormally warm temperatures increased the susceptibility of the plants to ever-present microorganisms (Rasmussen, 1977). The microorganism most commonly implicated was a slime-mold-like protist, *Labyrinthula* (Renn, 1934; Cottam and Addy, 1947), identified as *L. macrocystis* Cienk. (Young, 1943). In the decades since the first reports of the wasting disease there has been significant organism-specific research on *Labyrinthula* (Pokorny, 1967; Olive, 1975; Porter, 1988). The early work during the disease period was not conclusive because the necessary methods for axenic culture of *Labyrinthula* had not been developed (Renn, 1936; Johnson and Sparrow, 1961). Although axenic cultures of *Labyrinthula* (Watson and Ordal, 1957) were developed in the 1950s, tests of Koch's postulates were never attempted.

The current eelgrass wasting disease, first reported in the Great Bay Estuary, New Hampshire (Short *et al.*, 1986), occurs in two stages: (1) the initial infection of eelgrass leaves; and (2) the subsequent mass mortality of eelgrass. The infection is characterized by dark necrotic lesions on both young and old eelgrass leaves. The infection has now spread throughout the Great Bay Estuary, but the complete dieoff of eelgrass beds is restricted to local areas. Like the disease of the 1930s, the current epidemic is limited in the estuary by salinity; eelgrass growing in low salinity waters is less susceptible to infection. The decline is not universal; many areas showing infection symptoms as yet demonstrate no mass mortality.

The infection of eelgrass with the characteristic symptoms of the wasting disease is now widespread along the Atlantic coast of North America. Eelgrass collected in the summers of 1986 and 1987 from numerous sites between Nova Scotia, Canada,

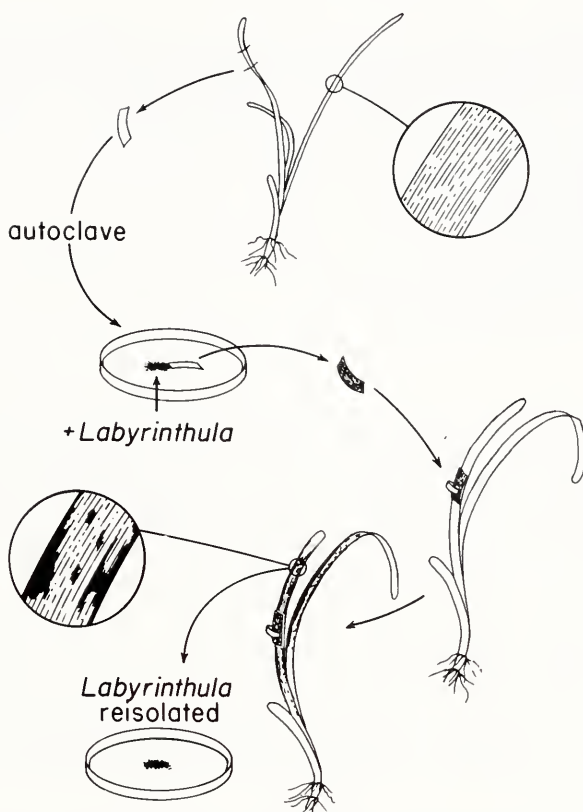


FIGURE 2. Procedure for laboratory infection of eelgrass, *Zostera marina* L., by *Labyrinthula*. Sterilized 1 cm pieces of uninfected, green eelgrass leaves were invaded with an axenic culture of *Labyrinthula* and then attached to a leaf of a healthy, green eelgrass shoot growing in an individual incubation flask. When a pathogenic strain was thus tested, the necrotic, blackened patches symptomatic of the wasting disease appeared on the eelgrass leaves within 14 h on some and within 24 h on all the plants. *Labyrinthula* was reisolated from the diseased leaves, thus satisfying Koch's postulates.

and Connecticut, USA, showed the infection (Fig. 1). Additionally, in 1986 infected eelgrass plants were found in Roscoff, France, and in Friday Harbor, Washington, and Beaufort, North Carolina, USA. The simultaneous occurrence of the wasting disease on both sides of the Atlantic is reminiscent of the reports of the 1930s disease (Fisher-Piette *et al.*, 1932; Huntsman, 1932; Cotton, 1933; Lewis and Taylor, 1933; Peterson, 1933; Taylor, 1933). The appearance of the disease on the Pacific coast was reported in the late 1930s (Young, 1938), with significant eelgrass decline evident in 1941 (Moffit and Cottam, 1941).

Our recent research has concentrated on determining the cause of the current eelgrass disease. Following Koch's postulates, we have successfully identified the causal agent to be a pathogenic strain of *Labyrinthula* (Fig. 2). We have regularly isolated this strain of *Labyrinthula* from diseased eelgrass leaves from Great Bay, New Hampshire and also from Beaufort, North Carolina and Friday Harbor, Washington. The pathogenic strain has never been isolated from healthy, green eelgrass

TABLE I

Labyrinthula infection experiments on eelgrass (*Zostera marina*) in laboratory culture

<i>Labyrinthula</i> source: Substrate, location	Number of replicates	Percent infection
Diseased eelgrass, Great Bay, NH	33	100
Diseased eelgrass, Puget Sound, WA	8	100
Diseased eelgrass, Beaufort, NC	4	100
Healthy eelgrass, Beaufort, NC	4	0
<i>Spartina</i> , Sapelo Is., GA	6	0
Codium drift, Weekapaug, RI	9	0
Mangrove leaf, Longboat Key, FL	7	0

leaves. Both pathogenic and non-pathogenic strains of *Labyrinthula* were isolated and then grown in pure culture using previously described procedures (Porter, 1988). The eelgrass infection experiments were performed in laboratories at both the University of Georgia and the University of New Hampshire (Table I). Every shoot exposed to the pathogenic strain of *Labyrinthula* (45 shoots of a total of 45) exhibited the disease symptoms. None of the nine control shoots, which were treated identically, but without *Labyrinthula* in the sterilized inoculum leaf piece, showed disease symptoms; all remained healthy. During the first week, the infection spread quickly, with the dark patches increasing to 3–4 cm lesions along the inoculated leaves. After two weeks, inoculated leaves were completely black or brown and, on most shoots, the infection had spread to other leaves. After three weeks, several of the infected shoots were completely brown and dead, while on other shoots the spread of the infection stopped; the growth of all the infected plants was greatly reduced relative to the control plants. *Labyrinthula* was reisolated from the infected leaves, thus satisfying Koch's postulates.

For the present study, four other strains of *Labyrinthula* were isolated from green eelgrass leaves and various other marine plants. When these strains were tested for pathogenicity, as described above, none of the 26 inoculated plants produced any signs of the disease symptoms (Table I).

It is significant that we found both pathogenic and non-pathogenic strains of *Labyrinthula* widely distributed in estuarine environments. It is possible that these represent different species, but the present taxonomic understanding of the species *Labyrinthula* is poor at best (Olive, 1975; Porter, 1988). A critical monograph of the genus is clearly needed.

Despite the widespread infection of eelgrass with the wasting disease, there is as yet relatively little documented evidence of disease-related declines. As mentioned above, the carefully monitored decline in the Great Bay Estuary has expanded (Short *et al.*, 1986). Since 1984, entire eelgrass beds have died and large portions of other beds have disappeared. A nearly complete decline of eelgrass at Cape Ann, Massachusetts in 1984 (Dexter, 1985), has been linked to disease through subsequent sampling of a few remnant eelgrass plants which proved to be infected.

Other declines in eelgrass have been reported. Loss of eelgrass from estuaries in both North America and Europe has been shown to result from pollution of coastal regions (Jones and Tippie, 1983; Kemp *et al.*, 1983; Neinhuis, 1983; Orth and Moore, 1983). Elsewhere in the world, other seagrasses have also experienced pollution-related declines (Cambridge and McComb, 1984). Loss of eelgrass was reported from several other areas along the east coast of the U. S. in 1986, including Buzzards Bay

and Cape Cod, Massachusetts, where the cause was pollution and Great South Bay, New York, where the cause was shading by a plankton bloom. Although the wasting disease has also been discovered in some of these locations, there is no evidence that it has contributed to any of these declines. However, we believe that the combined effects of the wasting disease and pollution could devastate eelgrass populations.

Whether the current eelgrass wasting disease will produce a catastrophic eelgrass decline is unknown. Although the current wasting disease has not yet caused a widespread decline of eelgrass populations, the disease poses a major threat to coastal fisheries, waterfowl populations, numerous marine habitats, and the health of estuarine environments. What has produced the recurrence of this widespread epidemic, what circumstances might bring this disease to the stage that devastates eelgrass populations, and what role environmental factors may play in this transition remain matters of conjecture and further investigation.

ACKNOWLEDGMENTS

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL
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ON DEVELOPMENTAL BIOLOGY
24-27 SEPTEMBER, 1987

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ABSTRACTS

In vivo properties of primary cilia in cultured kidney epithelial cells. S. S. BOWSER, K. E. ROTH, AND C. L. RIEDER (Wadsworth Ctr. Labs. & Res., NY State Dept. Health, Albany, NY).

A single primary (1°) cilium is generated from the parent centriole of the centrosome in most embryonic and terminally differentiated cell types. 1° cilia are oriented perpendicular to the dorsal cell surface, which makes them difficult to observe by conventional LM preparative methods. As a result, their properties and function remain unknown. To overcome this restriction, we grow kidney epithelial cell monolayers on plastic films. A side view of the monolayer is obtained by folding the film, cell-side out, and sandwiching it in a simple coverslip chamber. Along the folded edge, 1° cilia are perpendicular to the optical axis and readily imaged by real-time or time-lapse video-LM. 1° cilia appear as rigid, 0.25 μm -diameter rods and their average length is characteristic for each kidney cell line examined. 1° cilia show no beating motions but passively bend in response to flow. 1° cilia frequently possess one or more varicosities or swellings along the ciliary shaft. Time-lapse video LM reveals that these varicosities move bidirectionally between the ciliary base and tip. Same-cell correlative LM and high-voltage EM indicates that these varicosities are delimited by the ciliary membrane and contain a granular cytoplasmic matrix devoid of vesicles or other organelles. Our finding that cytoplasmic transport occurs along 1° cilia suggests they may serve as a conduit for exchange between the extracellular milieu and the nucleus.

Time-lapse photographic study of neural tube closure defects in the chick. K. T. BUSH, R. G. NAGELE, AND H. LEE (Department of Biology, Rutgers University, Camden, NJ and Department of Pediatrics, University of Medicine & Dentistry of New Jersey-School of Osteopathic Medicine, Camden, NJ).

A new method was described whereby early chick embryos were explanted using a modified New's technique [in which (1) avian Ringer's solution replaced Pannett-Compton saline and (2) the vitelline membrane with an adherent blastoderm was placed over the glass ring] and photographed with a Nikon Diaphot inverted microscope equipped with both phase-contrast optics and photomicrographic accessories maintained in an incubator. This method permits for the first time recording of sequential changes in the morphology of early chick embryos. Specifically, 4-somite stage embryos were explanted, grown in nutrient medium with or without a teratologic dose of xylocaine (lidocaine HCl, Elkins-Sinn), and were photographed at 2-3 hour intervals. We found, among other things, that a characteristic neural tube closure defect often seen in the brain region of xylocaine (200 $\mu\text{g}/\text{ml}$)-treated chick embryos was consequence of failure of the neural tube to withstand the tension generated by the rapidly expanding cephalic region which occurred when the corresponding controls had advanced to 11-somite stage.

Interspecific comparisons of the period gene. H. V. COLOT, G. PETERSEN, Q. YU, D. WHEELER, J. C. HALL, AND M. ROSBASH (Department of Biology, Brandeis University, Waltham, MA).

We have cloned and sequenced substantial portions of the *period* (*per*) gene from *Drosophila pseudoobscura*, *D. virilis*, and *D. simulans* as part of our continuing investigation into the function of the *per* gene

in circadian and ultradian rhythms. The *D. pseudoobscura* gene has been examined in the most detail and compared with the well-characterized *per* gene of *D. melanogaster*. The locomotor activity of *D. pseudoobscura* differs substantially in period length and strength, as well as overall pattern, from that of *D. melanogaster*. DNA sequence comparison between these two species shows blocks of diverged coding sequence interspersed with blocks of conserved coding sequence. Surprisingly, the TG-repeat region of *D. melanogaster* is replaced in *D. pseudoobscura* by a longer, totally different repeat that is preceded by only a few TG and SG pairs. (Note that deletion of most of the TG region of *D. melanogaster* affects the species-specific courtship song rhythm but not the circadian rhythm.) Therefore, the TG repeat is probably not a series of (obligatory) glycosaminoglycan attachment sites but may confer species-specific properties on the protein. Experiments involving cross-species transformation of the *per* gene have been initiated.

Pattern formation with Fusarium illustrates a principle for generation of cell pattern. J. DAS AND H. BUSSE (Biochem. Inst. Med. Fak., Univers. Kiel, GFR and Harvard Medical School, Dept. Physiology & Biophys., Boston, MA).

The fungus *Fusarium* usually grows by extending its mycelium outwards. If hyphae try to grow into a region already occupied by the mycelium of a neighboring fungus, then either of the two following processes can occur: (1) the hyphae do not recognize each other and intermingle; or (2) the hyphae recognize that the neighboring region is already occupied and stop growing.

In the second case, the pattern formed in the plane of growth can be understood in terms of a specific distance measure. A distance measure is a mathematical function which allows an object (a hypha in our case) to determine its distance relative to other objects in the neighborhood. If the distance measure reaches a preset limit a decision is made. In our example: hyphae stop to grow. That distance measures can produce spatial pattern is illustrated for *Fusarium*. It is likely that patterns in differentiating animal tissue can also be described by distance measures.

Integrin structure, function, and developmental expression. D. W. DESIMONE, M. A. STEPP, R. PATEL, E. MARCANTONIO, AND R. O. HYNES (Center for Cancer Research, M.I.T., Cambridge, MA).

The integrins are a family of cell-surface receptor complexes that participate in a variety of cell-cell and cell-extracellular matrix interactions, which are involved in morphogenesis, cell migration, hemostasis, oncogenesis, and immune function. Integrins exist as heterodimers made up of distinct α and β subunits. We have isolated cDNA clones of integrin subunits from several species. Comparisons among the cDNA sequences of β subunits from *Xenopus laevis*, chicken, mouse, and human reveal that the integrin family can be organized into at least three classes. We estimate that the three classes of β subunits diverged from a common ancestral gene at an early stage of metazoan evolution. Functional heterogeneity within integrin classes is probably conferred by a large number of distinct, yet structurally related, α subunits. The structural relatedness of the integrin receptors will be discussed in view of results obtained by Northern and Southern blot hybridization analyses, cDNA sequencing, and immunologic cross-reactivity. Similar approaches are being used to determine the developmental expression and functional significance of the integrins during amphibian embryogenesis.

Identification of a calcium-calmodulin dependent protein kinase associated with the sea urchin mitotic apparatus. J. H. DINSMORE AND R. D. SLOBODA (Dept. of Biological Sciences, Dartmouth College, Hanover, NH 03755).

Mitotic apparatuses (MAs) isolated from the sea urchin, *Lytechinus pictus*, were assayed for protein kinase activity; phosphorylation of endogenous MA substrates was determined by SDS-polyacrylamide gel electrophoresis and autoradiography. MAs were isolated by the Salmon (*Methods Cell Biol.* 25: 71-102, [1982]) detergent lysis method with the following modifications: embryos were treated with 3% hexylene glycol for 3 min prior to lysis; 50 μ M GTP and protease inhibitors were included in the lysis buffer; and MAs were washed two times in 50 mM PIPES, pH 6.9 plus 10 μ M taxol to obtain the final MA preparation. Isolated MAs were incubated with [γ - ^{32} P]ATP, Mg^{2+} , and one of the following: cAMP; cGMP; Ca^{2+} ; calmodulin; Ca^{2+} and calmodulin; Ca^{2+} , calmodulin and calmidazolium. Specific phosphorylation of only a single protein of Mr 62 kD occurred when both Ca^{2+} and calmodulin were present. Incubation of intact, birefringent MAs with 40 μ M ATP plus 0.3 μ M calmodulin resulted in an increased rate and extent of depolymerization of MA microtubules in response to micromolar Ca^{2+} . In the absence of either ATP or calmodulin this effect of Ca^{2+} on microtubule stability was not observed. Finally, *in vivo* phosphorylation experiments showed that protein phosphorylation in the embryo rose and fell with the cell cycle, with peak

levels of phosphorylation occurring at the metaphase-anaphase transition. The results suggest that specific Ca^{2+} -calmodulin dependent protein phosphorylation affects MA stability, and may be important in controlling the metaphase-anaphase transition *in vivo*. [Supported by NSF BNS85-03597 to RDS and a R. M. Cramer Graduate Fellowship and Sigma Xi Grant-in-Aid to JHD]

Inducible c-myc overexpression and F9 teratocarcinoma stem cell differentiation.

C. A. DIONNE AND L. J. GUDAS (Division of Cancer Genetics, Dana-Farber Cancer Institute and Pharmacology Department, Harvard Medical School, Boston, MA).

The retinoic acid-induced terminal differentiation of F9 teratocarcinoma stem cells is accompanied by a very early and rapid decrease in c-myc proto-oncogene expression. We have isolated several F9 cell lines which have stably integrated a tightly regulated, highly inducible c-myc expression vector. The morphological and biochemical differentiation of these transfected cell lines was similar to that of F9 wild type cells despite continuous induced c-myc overexpression throughout the differentiation period. In addition, c-myc overexpression did not prevent the decreased rate of cellular proliferation in response to retinoic acid. Our results argue that the previously reported dominant effects of c-myc on differentiation and cellular proliferation are not observed in embryonic stem cells such as F9 teratocarcinoma stem cells.

Nucleoprotein complexes that regulate gene expression in adipocyte differentiation:

direct participation of c-fos. ROBERT J. DISTEL, HYU-SUNG RO, BARRY S. ROSEN, DOUGLAS L. GROVES, AND BRUCE M. SPIEGELMAN (Dana-Farber Cancer Institute and Department of Pharmacology, Harvard Medical School, Boston, MA).

Adipocyte differentiation is accompanied by the transcriptional activation of many new genes, including a putative lipid binding protein termed adipocyte P2 (aP2). The aP2 gene contains a regulatory element (FSE2) 124 bases 5' to its start of transcription. This DNA sequence binds nuclear factors in a sequence-specific fashion as determined by its altered mobility in gel retardation assays. Deletion analysis of promoter-linked transfection assays and competition of these constructions in cells with synthetic FSE2 elements suggests that trans-acting factors bind in this region and act as negative regulators of aP2 gene activity in preadipocytes. c-fos appears to participate directly in this nucleoprotein complex, as demonstrated by the ability of antibodies to c-fos to disrupt specific binding of factors and the FSE2 sequence but not to factor-binding sequences from several other genes. Antibodies to c-fos specifically immunoprecipitate protein complexes covalently bound to FSE2 DNA via UV cross-linking.

Intracellular patterning and the problem of assembly. GARY W. GRIMES (Department of Biology, Hofstra University, Hempstead, NY 11550).

Ciliated protozoa typically are described on the basis of their highly asymmetric pattern of the ciliature. However, we have microscopically induced a ciliate possessing bilateral symmetry which is stably inherited during asexual (and presumably sexual) reproduction. Because these bilaterally symmetrical cells are essentially "Siamese Twins" fused side-by-side, with one half the mirror image of the other, one would predict that the asymmetry of the individual ciliary structures would correspondingly be mirror-imaged. However, they are not. Rather, they are assembled typically (*i.e.*, not mirror-imaged) and patterned in a mirror-imaged manner, or they are assembled in an inverted fashion but outwardly organized in a mirror-imaged pattern in order to correspond to the global asymmetry of the mirror-imaged half of the cell. These data suggest the presence of a global patterning mechanism imposed on the cortex of these ciliates to which the individual elements of the ciliature *must* respond during their assembly. Constraints on the way in which the ciliature *can* be assembled thus determines whether or not they are assembled in a "typical" or inverted manner. The general principles of "directed assembly" and "directed patterning" are emphasized by these studies. Supported by Research Grants from the NSF and Hofstra University.

Cell surface reorganization in the fertilized egg of the zebrafish. N. H. HART AND J. S. WOLENSKI (Department of Biological Sciences, Rutgers University, New Brunswick, NJ).

The time-course of surface reorganizational events and their sensitivity to microfilament inhibitors in the fertilized egg of the zebrafish (*Brachydanio*) were studied with light and electron microscopy. A single

sperm binds to a predetermined site on the egg oolemma within 5 s of insemination. A distinct fertilization cone formed at this site between 45–60 s. Complete incorporation of sperm head, midpiece, and a portion of the flagellum occurred between 60 and 120 s. The second polar body formed by 4 min near the site of gamete fusion. Cortical granule exocytosis, initiated beyond the site of gamete union by 30 s, was completed by 4–5 min. The reaction in the vicinity of the fertilizing sperm was visible by 60 s. Freeze fracture analysis showed higher IMP density on the P leaflet of the egg plasma membrane than on the P face of the cortical granule membrane. These differences persisted after cortical granule breakdown, suggesting that the two membrane domains do not mix rapidly. Cortical granule exocytosis and fertilization cone formation were unaffected by either cytochalasin B or D (10 $\mu\text{g}/\text{ml}$) treatment. Sperm incorporation was inhibited by either 10 $\mu\text{g}/\text{ml}$ CB or 50 $\mu\text{g}/\text{ml}$ CD. Sperm entry into the egg appears to require actin polymerization.

Molecular studies of biological rhythms in Drosophila. F. R. JACKSON AND K. J. ELLIOTT (Worcester Foundation for Experimental Biology, Shrewsbury, MA).

The fruitfly *Drosophila* is an excellent model system for genetic studies of biological clocks. As a consequence, several *Drosophila* genes have been identified that determine the properties of biological oscillators. One of these genes, *period* (*per*), encodes a proteoglycan-like glycoprotein whose function is currently being investigated in several laboratories. We have recently employed *per*-gene probes to identify and isolate segments of *Drosophila* genomic DNA containing related sequences. One of these 'per-homologous' clones appears to encode a proteoglycan which contains Thr-Gly repeats similar to those seen in the *per* protein. Experiments are underway to determine the relevance of this 'per-homologous' gene for the development and/or maintenance of circadian rhythms.

Another *Drosophila* clock gene, *Andante*, maps to chromosomal region 10E and lengthens periods of circadian and ultradian rhythms. We have cloned DNA sequences from the 10E region, and initiated a chromosomal walk towards the *And* locus. Chromosomal breakpoints which flank the *And* gene are currently being localized in cloned sequences to define the physical limits of the locus.

Isolation and characterization of an mRNA sequence (ERA-1) exhibiting a rapid and protein synthesis independent induction during the retinoic acid-induced differentiation of teratocarcinoma stem cells. G. J. LAROSA AND L. J. GUDAS (Program on Cell and Devel. Biology, Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA).

Vitamin A and its derivatives (retinoids) exhibit profound effects on the proliferation and differentiation of many cell types. F9 teratocarcinoma stem cells, which differentiate into non-tumorigenic primitive endoderm cells in response to retinoic acid (RA), serve as an excellent *in vitro* model for molecular studies of cellular differentiation and the mechanism by which RA can set this complex process into motion. In order to begin to analyze events which occur rapidly after RA treatment of F9 cells, we have constructed a cDNA library from RNA of F9 cells treated for 8 h with RA. By screening with a cDNA probe enriched for RA-induced sequences by subtractive solution hybridization, we have been able to isolate a cDNA clone for an RNA which exhibits the properties of a primary target of RA action. This gene, *Early Retinoic Acid-1* (ERA-1), encodes a 2.2 kb polyadenylated RNA which is rapidly induced by RA, in a dose-dependent manner, both in the absence or presence of protein synthesis inhibitors. The isolation of this gene sequence demonstrates that RA can influence gene expression very rapidly and also provides us with an important tool to further analyze the initial intracellular action of RA in this F9 differentiation model system.

Cellular interactions during amphibian gastrulation. J. LEBLANC (The College of Staten Island, CUNY, NY), M. YODER AND I. BRICK (New York University, NY).

Epithelial movement during amphibian gastrulation is responsible for much of the reorganization that establishes the primitive body plan. If cells are to form an epithelium and then exhibit epithelial translocations, sufficient intercellular cohesion, adhesions along their adjacent edges, would be required to maintain integrity. Cellular interactions at the dorsal and lateral lip areas of the blastopore in *Rana pipiens* embryos at various stages during gastrulation have been investigated by TEM.

Areas of unspecialized apposition and desmosomes were observed between adjoining cell surfaces (1) of lip cells lining the blastoporal groove, (2) of lip cells at the surface of the groove and underlying lip cells, (3) of cells of the yolk plug lining the blastoporal groove, (4) of yolk plug cells at the surface of the groove and underlying yolk plug cells, and (5) of lip and yolk plug cells. The cellular arrangements and intercellular

junctions suggest that the layer of lip cells and the layer of yolk plug cells that line the blastoporal groove are both an epithelium.

Primordial germ cells of day 5 chick embryos. B. Y. LEE, K. T. BUSH, R. G. NAGELE, AND H. LEE (Department of Pediatrics, University of Medicine & Dentistry of New Jersey-School of Osteopathic Medicine, Camden, NJ and Department of Biology, Rutgers University, Camden, NJ).

Primordial germ cells (PGCs) of the chick are initially identifiable with certainty in the germinal crescent area of the definitive streak stage embryo. As embryonic development proceeds, most PGCs migrate to the area vasculosa, enter blood vessels, and then reach the gonads. By day 5 of development, nearly all PGCs, which will eventually settle in the gonads, are already in the gonads. These PGCs are distinguishable from somatic (germinal epithelial) cells in that they have well-defined cell boundaries, numerous periodic acid Schiff-positive granules, prominent lipid droplets, ring-shaped Golgi complex, and fragmented nucleoli. They appear to relocate themselves in an ameboid fashion within the gonads. Of the several methods used for isolating PGCs from the gonads, treatment with 0.2% collagenase-0.1% trypsin inhibitor in calcium- and magnesium-free Hanks' balanced salt solution followed by the discontinuous Percoll density-gradient centrifugation was found to be most effective. Isolated PGCs, when grown *in vitro*, retained both their distinguishing features and the ability to migrate actively.

Leukemic viral expression with induced changes in hematopoietic microenvironment. G. P. LEONARDI, M. MANTHOS, J. LOBUE, D. ORLIC, AND J. MITRA (Dep't of Biology, New York Univ., New York, NY and Dep't of Anatomy, N. Y. Medical College, Valhalla, NY).

A transplantable, granulocytic leukemia has been established from BALB/c mice inoculated with a variant of Rauscher Leukemia Virus that normally induces severe derangement of erythropoiesis (RLV-A; LoBue *et al.*, 1972). Six weeks before, and following inoculation of this erythroblastic virus, these mice were kept at elevated red cell volumes by injection of 75% packed red cells (*i.e.*, hypertransfusion) every 7 days. Such hypertransfusion in mice eliminates red cell formation and alters the microenvironment making it more suitable for granulopoiesis. Development of the "typical" RLV-A pathogenesis resulting in erythroblastosis and fatal anemia was not observed in these animals. Instead, the appearance of massive numbers of leukocytic elements including transplantable monomyelocytic leukemic cells was seen in four of the six animals by 18-40 weeks post-viral inoculation. Hypertransfused control animals did not exhibit these reactions. Hypertransfusion eliminates production of red cells and their precursors, and modifies bone marrow stromal cells thus changing the microenvironment (Brookoff and Weiss, 1982). Sustained hypertransfusion by (1) eliminating virally susceptible erythroid cells or (2) modifying the hematopoietic microenvironment or (3) as a consequence of both resulted in the expression of a different viral oncogenic expression. The authors acknowledge the generous financial support of Dr. and Mrs. P. C. Chan for these studies.

Interleukin-2 (IL-2) distribution in adult newts (Notophthalmus viridescens) during regeneration and following non-amputational wounding. M. F. LOMBARD¹ AND R. E. SICARD² (¹Department of Biology, Regis College, Weston, MA; and ²Department of Pediatrics, Rhode Island Hospital, Providence, RI).

A role for the immune system in epimorphic regeneration has been proposed, but not proved. Accordingly, [³H]-IL-2 (a potent lymphokine that modulates immune response through its actions on macrophages and lymphocytes) was used to explore changes in distribution and availability of IL-2 responsive cells during regeneration and after non-amputational wounding. IL-2 content was reduced in major hemopoietic organs 2-8 days after trauma. Progressive increases, to control levels, occurred in livers of both groups; however, IL-2 content remained depressed in spleens of regenerating, but not "wounded," animals between 15-21 days post-trauma. During this same period, IL-2 content was reduced peripherally, as reflected by decreased content in non-traumatized forearms. Initially, IL-2 content of non-amputational wound sites was low, but progressively increased from 8-21 days post-trauma. In contrast, IL-2 content of stumps and blastemas decreased between 2-21 days postamputation. The apparent pattern of availability and distribution of IL-2 responsive cells during regeneration differed from that seen during repair of non-amputational wounds. These data are consistent with immunological influence on regeneration and suggest that further exploitation of IL-2 as a probe might help elucidate that role.

Sea urchin H2A.F/Z: an unusually conserved H2A variant gene. ROBERT McISAAC, HEIDI MILLER, CAROL A. BRENNER, CATHERINE NOCENTE-MCGRATH, SUSAN FRANCIS, AND SUSAN G. ERNST (Department of Biology, Tufts University, Medford, MA).

Sea urchin development is distinct in its sequential and overlapping use of multiple histone classes. In addition, variants have been isolated which are differentiation specific. We have identified a cDNA clone that encodes an H2A variant histone gene which is distinct from any class of sea urchin histones. The coding region is 74% homologous to the chicken H2A.F gene and a remarkable 97% conservation exists when comparing the putative amino acid sequences. Developmental expression does not coincide with any embryonic histone class and the message has been found in all adult tissues examined. The 1.7 kb transcript contains an unusually long 3' untranslated region and binds to oligo dt cellulose. In contrast to the early gene set, this sequence is present at only a few copies per haploid genome. Both the sea urchin and the chicken gene encode a protein related to the replication-independent protein H2A.Z found in vertebrates. This protein is present at a level of 1–10% of the total H2A. Its low abundance, highly conserved nature, and broad phylogenetic distribution all suggest a unique role for this protein in chromatin organization.

This work was supported by a Biomedical Research Grant and Tufts University Faculty Research Award to Susan G. Ernst.

Luminescence proteins exhibit circadian rhythms but their mRNAs do not. D. MORSE, P. MILOS, E. ROUX, AND J. W. HASTINGS (Biological Labs, Harvard University, Cambridge, MA 02138).

At least three components of the bioluminescent system in *Gonyaulax polyedra* are under circadian regulation: the substrate (luciferin), the luciferin binding protein (LBP), and the enzyme luciferase. LBP cDNA was isolated by immunological screening from a cDNA library subcloned into an expression vector. Its identity was confirmed by *in vitro* translation of hybrid selected mRNA. Northern hybridization to mRNAs isolated at 8 different time points spanning a 24-hour period showed that the amounts of the LBP mRNAs were invariant with time. A putative luciferase cDNA has also been isolated and experiments similarly indicate constant levels of the corresponding mRNAs. In a complementary approach, *in vitro* translation of the mRNAs used in the Northern experiments showed equal synthesis of LBP at all time points. These results indicate that circadian control of the amount of binding protein is exerted at the translational rather than the transcriptional level.

The guinea pig sperm surface protein PH-20 is anchored in the membrane by a phosphatidylinositol lipid. B. PHELPS, P. PRIMAKOFF, D. E. KOPPEL, AND D. G. MYLES. (Dept. of Physiology, University of Connecticut Health Center, Farmington, CT).

PH-20 is an integral protein of the guinea pig sperm plasma membrane, for which there is evidence for a role in sperm-zona pellucida binding [Primakoff *et al.*, *J. Cell Biol.* **101**: 2239 (1985)]. PH-20 is uniformly distributed over the entire cell surface of testicular sperm, but is localized to the posterior head region of distal cauda epididymal sperm. Following the exocytotic acrosome reaction the plasma membrane becomes contiguous with the inner acrosomal membrane and PH-20 migrates to the anterior head region [Myles and Primakoff, *J. Cell Biol.* **99**: 1634 (1984)]. Measurement of the diffusion coefficient of PH-20 using the technique of fluorescence recovery after photobleaching (FRAP), show that PH-20 diffusion on testicular sperm is highly restricted within the plane of the bilayer, while PH-20 on acrosome-reacted sperm is freely diffusing. At the intermediate stage, acrosome-intact cauda epididymal sperm, the protein diffuses at a rate between these two extremes; it is mobile but not freely diffusing. Exposure of testicular sperm, acrosome-intact, and acrosome-reacted epididymal sperm to a phosphatidylinositol-specific phospholipase C results in the release of PH-20 from the cell surface. Thus, a cell that is no longer capable of protein synthesis is able to modulate, during its differentiation, both the surface distribution and the rate of diffusion of an integral membrane protein that is anchored in the bilayer by a lipid. Supported by GM23585 to D.E.K. and NIH HD16580 to D.G.M.

Molecular analyses of early neural pattern decisions in Xenopus. CAREY PHILLIPS
(Department of Biology, Bowdoin College, Brunswick, ME 04011).

Orientation of the *Xenopus* nervous system results from an interplay between the animal-vegetal axis, established during oogenesis, and intracellular rearrangements, apparently cued by the point of sperm entry. Many subsequent cellular pathway choices are necessarily involved in construction of a nervous system and these choices may be reflected by changes in molecular synthetic patterns. Several molecular probes have been constructed to use in assaying for early determinative events leading to neural differentiation. Epi 1, a monoclonal antibody, is being used as a molecular probe to distinguish between cells proceeding along a neural pathway from cells destined to become epidermis. Using the Epi 1 probe, we have determined that the position of the presumptive neural tissue is approximately, although reversibly, established by at least the third cleavage division. We have also determined that the information necessary for expression of the Epi 1 antigen is associated with the cortex of the uncleaved embryo. The molecular nature of this information is being addressed experimentally. We are now in a position to use Epi 1 and other molecular probes to study how a series of pathway choices might interact to produce the final position of neural structures.

Protein-DNA interactions on the 5' non-transcribed spacer of Tetrahymena thermophila rDNA. K. RIEKKI AND R. E. PEARLMAN (Department of Biology, York University, Toronto, Ontario, Canada, M3J 1P3).

The 5' non-transcribed spacer (NTS) of the rDNA of *T. thermophila* contains sequences specifying a number of important functions including bidirectional origin of replication, promotion of RNA polymerase I catalyzed transcription, nucleosome phasing, rDNA copy number control, and topoisomerase I cleavage. We have initiated studies to correlate structure with function and to look at sequence specific protein-DNA interaction in this region.

Various restriction fragments in the 5' NTS have extremely anomalous mobility when electrophoresed through polyacrylamide gels suggesting sequence directed bending. In the center proximal (650 bp) *TaqI-XbaI* fragment, the bending locus appears to be in the 3' portion of this fragment. Using crude nuclear protein extracts and an electrophoretic mobility shift assay to assess protein-DNA interaction, we have demonstrated protein-DNA interaction in this *TaqI-XbaI* fragment. Greater interaction and specificity appears to occur with the 5' region of this sequence. Binding of protein from the same nuclear extracts also occurs to the 420 bp *XbaI-XbaI* fragment. Competition for this binding with unlabelled DNA from wild type C3 and from the rmm 1 mutation (Larson *et al.*, Cell 47: 229-240 (1986)) suggests interesting and possibly functionally significant specificity of this interaction. DNase I footprint experiments and protein fractionation are in progress in attempts to further assess the specificity of these protein-DNA interactions.

Effect of photoperiod and melatonin in glucosaminidase activity during newt limb regeneration. M. RIVERA, R. APONTE, F. CALIMANO, AND F. VALLES (Cayey University College, Department of Biology, Cayey, P.R. 00633).

The effect of continuous light, continuous darkness, and melatonin on the activity of the lysosomal enzyme N-acetyl glucosaminidase during limb regeneration in the newt *Notophthalmus viridescens* was studied. Animals exposed to continuous light showed more enzyme activity than the controls during the first 12 days after amputation. In these animals the highest peak of activity was exhibited on day 7, whereas the controls showed the highest peak at day 5. The pattern of activity is the same in both groups. In animals exposed to continuous darkness, the highest peak is observed on day 10 after amputation; the activity pattern is delayed as compared with controls. Animals whose forelimbs were amputated and were injected with melatonin showed a decrease in enzyme activity. The highest peak was detected on day 12 after amputation. The relationship between darkness and melatonin is discussed. Part of this work is supported by NIH Grant 5 S14 RR02640.

Studies on a transplantable monomyelocytic leukemia and in the offspring from crosses between transplanted and normal BALB/c mice. E. RODRIGUEZ, J. MITRA, AND J. LOBUE. (Department of Biology, New York University, New York City, New York).

In vivo cytogenetical studies on a transplantable monomyelocytic leukemia (MML) initially induced in female BALB/c mice (Fredrickson *et al.*, *J. Natl. Cancer Inst.* **48**: 1597-1605, 1972) by the Rauscher leukemia virus (RLV) has revealed: (a) The presence of a marker deleted chromosome 18 in all somatic tissues examined (bone marrow, peripheral blood, and spleen) restricted to female transplanted cells; (b) a high degree of centromeric associations mainly in peripheral blood; and (c) in MML transplanted mice, chromosome #19 shows presence of NOR in addition to chromosomes 12, 15, 16, and 18, whereas control animals have NORs on chromosomes 12, 15, 16, and 18.

The disease has been shown to cause abnormalities in meiosis (Segenreich *et al.*, *Genetics* **113**: s20, 1986). Male MML transplanted animals have been mated at mid stage of the disease (third day) with normal females. Analysis of the offspring is presently being performed. Litter size and phenotypical characteristics of the offspring were normal. To date no significant cytogenetical abnormalities have been found in the somatic tissues of the offspring and hematological parameters on peripheral blood seem to be normal.

Localization of laminin and collagen IV transcription in mouse teratocarcinomas and embryos by in situ hybridization. M. B. ROGERS AND L. J. GUDAS (Dana-Farber Cancer Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA).

Mouse F9 teratocarcinoma cells can be induced in culture to form two distinct extra-embryonic cell types: parietal and visceral endoderm. F9 cells grown in monolayer and treated with retinoic acid (RA) differentiate into parietal endoderm while cells allowed to form aggregates in the presence of RA form embryoid bodies with an outer layer of visceral endoderm. Visceral endoderm is characterized by the synthesis of alpha-fetoprotein and the basement membrane components laminin and collagen IV. The transcription of these genes has been characterized by *in situ* hybridization of radioactive recombinant probes to frozen sections of embryoid bodies. In contrast to previous observations from other laboratories on basement membrane protein expression, laminin and collagen IV mRNAs were found in the outer cell layer of embryoid bodies whether or not RA was present. Additional experiments are extending the technique to sections of preimplantation mouse embryos.

Meiotic chromosome studies in BALB/c mice bearing a transplantable mono-myelocytic leukemia (MML) and in the offspring of normal females crossed with transplanted males. E. J. SEGENREICH, J. MITRA, AND J. LOBUE (Department of Biology, New York University, New York, NY).

Meiotic chromosome studies have been performed on BALB/c mice transplanted with mono-myelocytic leukemia (MML), an acute disease induced by a virally (RLV) transformed cell (Fredrickson *et al.*, *J. Natl. Cancer Inst.* **48**: 1597-1605, 1972). Results indicate that MML transplantation is associated with increases in meiotic chromosomal aberrations. Levels of aneuploidy, polyploidy, structural alterations, and precocious separations rise throughout the six day disease. Spermatogonial chromosomes also show increases in aneuploidy, polyploidy, and structural alterations. These mitotic chromosomes exhibit a higher degree of anomalies than those in other tissues of the leukemic animal (E. Rodriguez, unpub. data). Successful mating of normal females and mid-stage (day 3) MML transplanted males has produced phenotypically and, as of one year of age, hematologically normal litters. Preliminary studies of the offspring have revealed mostly insignificant changes in the frequencies of meiotic aberrations compared to the control litters. Only a slight increase in polyploidy is observed. Spermatogonial mitosis also shows slight rises in the level of polyploidy along with centromeric affinity. The nature of susceptibility to the pathological parameters of the disease as well as to meiotic disturbances, will be determined by MML transplantation into the offspring.

Spatial patterning of neuronal differentiation in the leech. M. SHANKLAND AND M. Q. MARTINDALE (Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA).

We have examined the spatial patterning of neuronal differentiation in the metameric nerve cord of the leech using an antibody to molluscan small cardioactive peptide B (SCP). In the adult, anti-SCP stains an unpaired interneuron that is present only on the right or left side of the segmental ganglion in abdominal segments 1–3 and 18–21. Both antibody staining and intracellular injection of Lucifer Yellow reveal that this neuron extends an axon through the connective nerve into adjacent ganglia. The unpaired neuron is equally likely to lie on either side of a given segment, but there is greater than 95% likelihood that it will be on alternate sides (*e.g.*, right:left) in successive segments. In the embryo, every abdominal ganglion initially has a bilateral pair of these neurons and both express SCP-like immunoreactivity. As development proceeds one member of the pair ceases to stain in segments 1–3 and 18–21, while both members of the pair cease to stain in segments 4–17. The right:left alternation observed in the mature pattern could arise because the unpaired neuron in one ganglion influences the right:left decision in adjacent ganglia through its interganglionic axon. We have obtained support for this idea by showing that the pattern of alternation is locally disrupted by transecting the embryonic nerve cord prior to the onset of asymmetry.

Atrial natriuretic peptide (ANP) levels during rat ontogeny. R. E. SICARD AND J. C. WERNER (Division of Pediatric Cardiology, Department of Pediatrics, Rhode Island Hospital, & Program in Medicine, Brown University, Providence, RI).

ANP is an important modulator of fluid/electrolyte and pressure homeostasis. Its role and mechanisms of action have been extensively explored in adults; however, adequate appreciation of the developmental physiology of ANP is limited. Accordingly, we have begun exploring ANP metabolism during rat development. Levels of immunoreactive ANP (ir-ANP) were determined by radioimmunoassay (reagents from Amersham) in amniotic fluids or plasma during the last trimester of pregnancy and the first 2 months of life. During the last trimester, amniotic fluids displayed a biphasic change in ir-ANP levels: rising from <10 fmol/ml (13 days gestation) to 31.4 ± 7.3 fmol/ml ($n = 29$; 18–19 days gestation), then falling to <10 fmol/ml ($n = 5$). Plasma concentrations of ir-ANP remained <10 fmol/ml during this time, then rose during the first month [25.9 ± 7.1 fmol/ml, $n = 21$] to approximately adult levels [22.3 ± 5.4 fmol/ml, $n = 29$]. These data suggest that significant changes in ANP metabolism and activity might be occurring during the perinatal period in rats.

Localization of a gene product in Drosophila that influences biological rhythms. K. K. SIWICKI, C. EASTMAN, M. ROSBASH, AND J. C. HALL (Biology Department, Brandeis University Waltham, MA 02254).

The *period* (*per*) gene of *Drosophila melanogaster* influences the period of circadian rhythms as well as an ultradian rhythm (period *ca.* 1 min) in the fly's courtship song. To begin to investigate how the gene regulates these complex behaviors, we used an antibody specific for the *per* protein to examine its anatomical distribution with immunocytochemical methods. Staining was detected in the eyes, optic lobes, and brain of wild type flies, but was not present in *per*[−] mutants. Rhythmic fluctuations in the intensity of the staining were apparent: the pattern was much more prominent at night than during the day. The day/night cycling was observed during entrainment (12 h light/12 h dark) and continued to cycle in constant darkness, suggesting that an endogenous circadian oscillator may regulate the expression of the *per* gene, which itself influences circadian rhythms. Supported by NS-07873 to K.K.S. and GM-33205 to M.R. and J.C.H.

Centrosomes in echinoderm development. G. SLUDER, F. J. MILLER, K. LEWIS, AND C. L. REIDER (Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545).

In animal cells, centrosomes are an ensemble of poorly understood organelles found at the ends of the mitotic or meiotic spindles. Centrosomes are required for the establishment of the bipolar spindle axis, chromosome orientation/alignment, chromosome movement, and the establishment of the cleavage furrow. We will briefly introduce the centrosome and then discuss the origin of the centrosomes used in the development of the echinoderm zygote. We demonstrate for sea urchin eggs that the centrosomes used in development come only from the sperm; the egg centrosome is apparently lost. We then describe our

characterization of centrosome inheritance in starfish eggs. This system is interesting because the egg is fertilized at meiosis I when it contains two functional centrosomes. However, only the male centrosome is used in development; the female centrosomes are functionally lost prior to first mitosis. We will describe ongoing experiments which seek to: (a) provide an understanding of what functional and structural aspects of the female centrosomes are lost; (b) show that male and female centrosomes have intrinsically different stabilities; (c) determine when, during the meiotic sequence, the female centrosomes are destabilized.

Mouse cellular retinoic acid binding protein: cloning, cDNA sequence and mRNA expression during the retinoic acid-induced differentiation of F9 wild type and RA-3-10 mutant teratocarcinoma cells. CAROL M. STONER AND LORRAINE J. GUDAS (Dana-Farber Cancer Institute and Dept. of Pharmacology, Harvard Medical School, Boston, MA).

Retinoic acid, a natural derivative of vitamin A (retinol), induces mouse F9 teratocarcinoma stem cells to differentiate into nontumorigenic parietal endoderm cells. The mouse cellular retinoic acid binding protein (CRABP) has been implicated in the mechanism of action of retinoic acid (RA). A mutant F9 teratocarcinoma cell line, RA-3-10, which possesses less than 5% of the wild type level of CRABP activity, fails to differentiate in response to RA. To study the role that CRABP plays in the RA-induced differentiation process, we cloned the mouse CRABP cDNA and determined its full-length sequence. Southern analysis of F9 genomic DNA hybridized to CRABP cDNA suggests that the CRABP gene is present as a single copy gene in the haploid genome and that the gene contains two introns. Northern analysis shows that the CRABP mRNA is a single, low abundant mRNA approximately 800 bases in length. The steady state CRABP mRNA level remains relatively constant during the RA-induced differentiation of F9 wild type cells. The CRABP mRNA level is approximately 20-fold lower in the RA-3-10 mutant stem cells than in F9 wild type stem cells.

Cyclins and the cell cycle of early clam embryos. K. I. SWENSON¹ AND J. V. RUDERMAN² (¹Dept. of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA; ²Dept. of Zoology, Duke University, Durham, NC).

Fertilized oocytes of the surf clam require new protein synthesis in order to complete meiosis. New protein synthesis is also required during each cell cycle of the early embryo for the completion of the mitotic divisions as well (Hunt and Ruderman, unpub.). Possible candidates for these M-phase inducing proteins are the cyclins A and B, the levels of which periodically accumulate and disappear as a function of the mitotic cell cycle (Evans *et al.*, 1983, *Cell* 37: 389; Swenson *et al.*, 1986, *Cell* 47: 861; Westendorf, unpub.). The suspected M-phase inducing role of cyclin A has been confirmed by showing that *Xenopus* oocytes, when microinjected with *in vitro* synthesized SP6 cyclin A mRNA, enter meiosis. The activity of cyclin B in this assay system is unknown. Recently we found that cyclin A was tightly associated with a kinase activity. We are interested in the functional properties and specificities of this kinase activity and its involvement, if any, in the ability of cyclin A to induce M-phase.

Molecular genetics of early embryogenesis in C. elegans. A. TELFER, D. LEVITAN, U. GIAMBARELLA, AND D. T. STINCHCOMB (Harvard University, Cambridge, MA).

The first division of embryogenesis in the nematode, *Caenorhabditis elegans*, produces blastomeres that display different cell division patterns and are determined to express different developmental potentials. Mutations in one class of maternal effect genes disrupt early development: the first division produces blastomeres that divide synchronously and fail to express certain differentiated cell types. These mutants are termed *par* for they are defective in the partitioning of germ line granules, they fail to segregate factors that control cell cycle rates, and they possibly fail to partition determinants of some cell types. We have identified and isolated restriction fragment polymorphisms (RFLPs) on either side of the *par-1* locus. DNA between the two markers has been cloned. We will delimit the *par-1* gene by identifying maternal transcripts and by microinjecting cloned DNA to assess its function. Similarly, a RFLP in or near the *par-2* gene has been identified and adjacent DNA has been isolated. Characterization of the *par* gene products may help us understand how early blastomeres become determined during development.

Retinoids and pattern formation in vertebrate limbs. CHRISTINA THALLER AND GREGOR EICHELE (Dept. of Physiology, Harvard Medical School, Boston, MA).

All-*trans*-retinoic acid induces striking digit pattern duplications when locally applied to the developing chick limb bud. Instead of the normal digit pattern (234) a mirror-symmetrical 432234 pattern can be specified. Hence, retinoic acid closely mimics posterior limb bud tissue (known as the zone of polarizing activity, ZPA) that causes very similar duplications when grafted to an anterior site of a host limb bud. This raises an intriguing possibility: that retinoic acid is a natural signalling substance involved in limb pattern formation. We discovered that chick limb buds contain endogenous retinoic acid and found that retinoic acid but not its biologically inactive precursor retinol, forms a concentration gradient across the limb anlage with a highpoint in the posterior domain of the limb bud, the part that contains the ZPA. Moreover, the amount of endogenous retinoic acid detected in the limb bud is the same as required to induce duplications when retinoic acid is provided exogenously. To examine whether retinoic acid is produced *in situ*, we locally applied its precursors, all-*trans*-retinol and all-*trans*-retinal, in radioactive form. Both compounds are metabolized: retinol to retinal and retinal to retinoic acid. These observations strengthen the hypothesis that retinoic acid is a local chemical mediator involved in the specification of the anteroposterior limb pattern.

Region specific expression of mouse homeo box genes. M. F. UTSET¹, A. AWGULEWITSCH², W. MCGINNIS³, AND F. H. RUDDLE^{1,2} (Departments of ¹Human Genetics, ²Biology, and ³Molecular Biophysics and Biochemistry, Yale University, P.O. Box 6666, New Haven, CT 06510).

Mammalian homeo box genes show extensive homology to *Drosophila* homeotic and segmentation genes. As a step toward determining their functions, we are studying the patterns of homeo box gene expression during mouse development. Results from a number of laboratories indicate that several mouse homeo box genes display region specific patterns of expression within the developing central nervous system and mesoderm. For example, Hox-2.1 expression within the central nervous system of the newborn mouse and the 13.5 day mouse embryo appears restricted to the medulla and spinal cord, whereas at the same stages Hox-3.1 expression is found within the spinal cord posterior to the third cervical vertebra (*Science* 235: 1379-1382). At earlier embryonic stages Hox-3.1 expression is also detected in a spatially restricted pattern in mesodermal tissues. These patterns of expression are consistent with the hypothesis that mouse homeo box genes perform region specific developmental functions akin to those of *Drosophila* homeotic genes.

Visualization of actin with rhodamine phalloidin in the zebrafish egg. J. S. WOLENSKI AND N. H. HART (Department of Biological Sciences, Rutgers University, New Brunswick, NJ).

The distribution of polymerized actin in zebrafish (*Brachydanio*) eggs was determined using rhodamine phalloidin and fluorescence microscopy. Unactivated eggs exhibited a prominent and continuous band of fluorescence just beneath the plasma membrane. Whole eggs showed little evidence of staining in the remaining cortical cytoplasm. However, staining was visible around individual cortical granules in cortical fragments prepared from formaldehyde-fixed cells. A prominent, narrow band of staining was also detected at the periphery of activated eggs. Continuity of this fluorescent layer was interrupted at sites of fusion between the egg plasma membrane and exocytosing cortical granules. Gaps in the fluorescence were also evident in eggs treated with cytochalasin B. Our results suggest that polymerized actin is present in cortices of eggs including the site of sperm entry.

Developmental regulation of three testis-specific genes during mouse spermatogenesis. P. C. YELICK, Y. KWON, P. A. BOWER, K. C. KLEENE, AND N. B. HECHT (Department of Biology, Tufts University, Medford, MA 02155).

Mouse spermatogenesis is the continuous process of development in which a progenitor germ cell differentiates into mature spermatozoa. We have identified three testis-specific genes, mouse protamine 1 (mP1), mouse protamine 2 (mP2), and mouse transition protein 1 (mTP1), all of which are expressed in the haploid phases of spermatogenesis. All three gene products are involved in the nuclear condensation events which occur during the sequential replacement of the nucleosomal histones by mouse protamines.

All three genes are very similarly regulated. mP1, mP2, and mTP1 genes are first transcribed in the round spermatid stage of spermiogenesis. All three mRNAs are translationally regulated in that they are first transcribed and then stored for up to eight days before they are translated. The translated forms of all three mRNAs, as present on polysomes, differ from the RNP-complexed forms in that the poly (A)+ tails are shortened by about 140 nucleotides. We also demonstrate the presence in rat and hamster of gene sequences and testis-specific mRNAs homologous to mP1 and mP2 cDNAs. Comparison of protamine 1 and 2 gene expression in rat, hamster, and mouse demonstrates variable expression of the protamine 1 and 2 genes that is also reflected in the protamine 1 and 2 content of hamster, rat, and mouse sperm.

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